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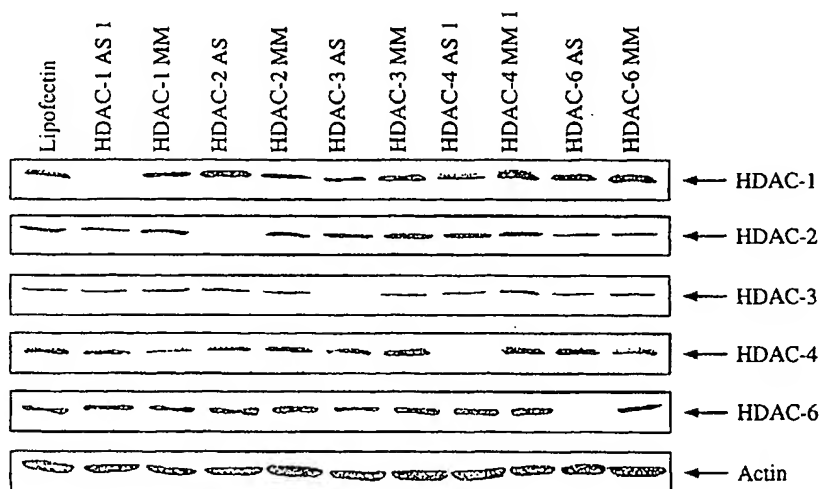
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(54) Title: **INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS**



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc - 50nM

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

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BACKGROUND OF THE INVENTIONField of the Invention

This invention relates to the fields of inhibition of histone deacetylase expression and enzymatic activity.

10

Summary of the Related Art

In eukaryotic cells, nuclear DNA associates with histones to form a compact complex called chromatin. The histones constitute a family of basic proteins which are generally highly conserved across eukaryotic species. The core histones, termed H2A, H2B, H3, and H4, associate to form a protein core. DNA winds around this protein core, with the basic amino acids of the histones interacting with the negatively charged phosphate groups of the DNA. Approximately 146 base pairs of DNA wrap around a histone core to make up a nucleosome particle, the repeating structural motif of chromatin.

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Csordas, *Biochem. J.*, 286: 23-38 (1990) teaches that histones are subject to posttranslational acetylation of the epsilon-amino groups of N-terminal lysine residues, a reaction that is catalyzed by histone acetyltransferase (HAT1). Acetylation neutralizes the positive charge of the lysine side chain, and is thought to impact chromatin structure. Indeed, Taunton *et al.*, *Science*, 272: 408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent regions of the genome.

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Recently, there has been interest in the role of histone deacetylase (HDAC) in gene expression. Sanches Del Pino *et al.*, *Biochem. J.* 303: 723-729 (1994) discloses a partially purified yeast HDAC activity. Taunton *et al.* (*supra*) discloses a human HDAC that is related to a yeast transcriptional regulator and suggests that this protein may be a key regulator of eukaryotic transcription.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation. Yoshida *et al.*, *J. Biol. Chem.* 265: 17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent inhibitor of mammalian HDAC. Yoshida *et al.*, *Cancer Research* 47: 3688-3691 (1987) discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

More recently, it has been discovered that the HDAC activity is actually provided by a set of discrete HDAC enzyme isoforms. Grozinger *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 96: 4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC1, HDAC2, and HDAC3 proteins are members of the first class of HDACs, and discloses new proteins, named HDAC4, HDAC5, and HDAC6, which are members of the second class of HDACs. Kao *et al.*, *Gene & Development* 14: 55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al.* *J. Bio. Chem.* 275:15254-13264 (2000) disclosed the newest member of the first class of histone deacetylases, HDAC-8. It has been unclear what roles these individual HDAC enzymes play.

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The known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone deacetylase isoform, and more or less inhibit all members of both

5 the histone deacetylase families equally.

Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to identify and inhibit specific histone deacetylase isoforms involved in tumorigenesis.

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BRIEF SUMMARY OF THE INVENTION

The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The inventors have discovered new agents that inhibit specific HDAC isoforms. Accordingly, in a first aspect, the invention provides agents that inhibit one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms. Such specific HDAC isoforms
15 include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. Non-limiting examples of the new agents include antisense oligonucleotides (oligos) and small molecule inhibitors specific for one or more HDAC isoforms but less than all HDAC isoforms.

20 The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the
25 invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

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In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding that histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In some
5 embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC isoform. In other embodiments, the oligonucleotide inhibits translation of the histone deacetylase isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Particularly preferred embodiments include antisense
10 oligonucleotides directed to HDAC-1 and/or HDAC-4.

In yet other embodiments of the first aspect, the agent that inhibits a specific HDAC isoform is a small molecule inhibitor that inhibits the activity of one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms.

15 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell, comprising contacting the cell with an agent of the first aspect of the invention. In other preferred embodiments, the agent is an antisense oligonucleotide. In certain preferred embodiments, the agent is a small
20 molecule inhibitor. In other certain preferred embodiments of the second aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the
25 invention further comprises contacting the cell with a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of one or more specific histone deacetylase isoforms. In still yet other preferred embodiments of the second aspect of the invention, the method comprises an agent of the first aspect of the invention which is a

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combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In
5 other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting
10 neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide which is combined with a pharmaceutically acceptable
15 carrier and administered for a therapeutically effective period of time. In certain preferred embodiments, the agent is a small molecule inhibitor which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments of the this aspect of the invention, cell proliferation
20 is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In other certain embodiments, the agent is a small molecule inhibitor of the first aspect of the invention which is combined with a pharmaceutically acceptable carrier and administered for
25 a therapeutically effective period of time. In still yet other preferred embodiments of the third aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred

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embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

- 5 In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone
- 10 deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that
- 15 inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is
- 20 a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In
- 25 certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of

5 differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other

10 certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense

15 oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting

20 neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for

25 a therapeutically effective period of time.

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In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide from the first aspect of the invention that inhibits expression of a specific histone deacetylase isoform, a small molecule inhibitor from the first aspect of the invention that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a small molecule that inhibits a DNA methyltransferase. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation, comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In certain embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In preferred embodiments, the cell proliferation is neoplasia. In still yet other preferred embodiments of the this aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram providing the amino acid sequence of HDAC-1, as provided in GenBank Accession No. AAC50475 (SEQ ID NO:1).

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Figure 1B is a schematic diagram providing the nucleic acid sequence of HDAC-1, as provided in GenBank Accession No. U50079 (SEQ ID NO:2).

10

Figure 2A is a schematic diagram providing the amino acid sequence of HDAC-2, as provided in GenBank Accession No. AAC50814 (SEQ ID NO:3).

15

Figure 2B is a schematic diagram providing the nucleic acid sequence of HDAC-2, as provided in GenBank Accession No. U31814 (SEQ ID NO:4).

20

Figure 3A is a schematic diagram providing the amino acid sequence of HDAC-3, as provided in GenBank Accession No. AAB88241 (SEQ ID NO:5).

25

Figure 3B is a schematic diagram providing the nucleic acid sequence of HDAC-3, as provided in GenBank Accession No. U75697 (SEQ ID NO:6).

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Figure 4A is a schematic diagram providing the amino acid sequence of HDAC-4, as provided in GenBank Accession No. BAA22957 (SEQ ID NO:7).

5 Figure 4B is a schematic diagram providing the nucleic acid sequence of HDAC-4, as provided in GenBank Accession No. AB006626 (SEQ ID NO:8).

10 Figure 5A is a schematic diagram providing the amino acid sequence of HDAC-5, as provided in GenBank Accession No. BAA25526 (SEQ ID NO:9).

15 Figure 5B is a schematic diagram providing the nucleic acid sequence of HDAC-5 as provided in GenBank Accession No. AB011172 (SEQ ID NO:10).

20 Figure 6A is a schematic diagram providing the amino acid sequence of human HDAC-6, as provided in GenBank Accession No. AAD29048 (SEQ ID NO:11).

Figure 6B is a schematic diagram providing the nucleic acid sequence of human HDAC-6, as provided in GenBank Accession No. AJ011972 (SEQ ID NO:12).

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Figure 7A is a schematic diagram providing the amino acid sequence of human HDAC-7, as provided in GenBank Accession No. AAF63491.1 (SEQ ID NO:13).

5 Figure 7B is a schematic diagram providing the nucleic acid sequence of human HDAC-7, as provided in GenBank Accession No. AF239243 (SEQ ID NO:14).

10 Figure 8A is a schematic diagram providing the amino acid sequence of human HDAC-8, as provided in GenBank Accession No. AAF73076.1 (SEQ ID NO:15).

15 Figure 8B is a schematic diagram providing the nucleic acid sequence of human HDAC-8, as provided in GenBank Accession No. AF230097 (SEQ ID NO:16).

20 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-1 AS1 antisense oligonucleotide on HDAC-1 mRNA expression in human A549 cells.

25 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-2 AS antisense oligonucleotide on HDAC-2 mRNA expression in human A549 cells.

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Figure 9C is a representation of a Northern blot demonstrating the effect of HDAC-6 AS antisense oligonucleotide on HDAC-6 mRNA expression in human A549 cells.

- 5 Figure 9D is a representation of a Northern blot demonstrating the effect of HDAC-3 AS antisense oligonucleotide on HDAC-3 mRNA expression in human A549 cells.

- 10 Figure 9E is a representation of a Northern blot demonstrating the effect of an HDAC-4 antisense oligonucleotide (AS1) on HDAC-4 mRNA expression in human A549 cells.

- 15 Figure 9F is a representation of a Northern blot demonstrating the dose-dependent effect of an HDAC-4 antisense oligonucleotide (AS2) on HDAC-4 mRNA expression in human A549 cells.

- 20 Figure 9G is a representation of a Northern blot demonstrating the effect of an HDAC-5 antisense oligonucleotide (AS) on HDAC-5 mRNA expression in human A549 cells.

- Figure 9H is a representation of a Northern blot demonstrating the effect of an HDAC-7 antisense oligonucleotide (AS) on HDAC-7 mRNA expression in human A549 cells.

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Figure 9I is a representation of a Northern blot demonstrating the dose-dependent effect of HDAC-8 antisense oligonucleotides (AS1 and AS2) on HDAC-8 mRNA expression in human A549 cells.

5 Figure 10A is a representation of a Western blot demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype protein expression in human A549 cells.

10 Figure 10B is a representation of a Western blot demonstrating the dose-dependent effect of the HDAC-1 isotype-specific antisense oligo (AS1 and AS2) on HDAC isotype protein expression in human A549 cells.

15 Figure 10C is a representation of a Western blot demonstrating the effect of HDAC-4 isotype-specific antisense oligonucleotide (AS2) on HDAC isotype protein expression in human A549 cells.

20 Figure 11A is a graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.

Figure 12A is a graphic representation demonstrating the effect of HDAC-1 AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

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Figure 12B is a graphic representation demonstrating the effect of HDAC-8 specific AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

5 Figure 13 is a a graphic representation demonstrating the cell cycle blocking effect of HDAC specific antisense oligonucleotides on human A549 cancer cells.

10 Figure 14 is a representation of an RNase protection assay demonstrating the effect of HDAC isotype-specific antisense oligonucleotides on HDAC isotype mRNA expression in human A549 cells.

15 Figure 15 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 AS1 antisense oligonucleotide induces the expression of the p21 protein.

20 Figure 16 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-1 antisense oligonucleotides (AS1 and AS2) represses the expression of the cyclin B1 and cyclin A genes.

25 Figure 17 shows plating data demonstrating the ability of antisense oligonucleotides complementary to HDAC-1 to inhibit growth in soft agar of A549 cells far more than can antisense oligonucleotides complementary to HDAC-2, HDAC-6 or mismatched controls.

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Figure 18 is a representation of a Western blot demonstrating that treatment of human A549 cells with the small molecule inhibitor Compound 3 (Table 2) induces the expression of the p21 protein and represses the expression of the cyclin B1 and cyclin A genes.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and reagents for inhibiting specific histone deacetylase isoforms (HDAC) by inhibiting expression at the nucleic acid level or protein activity at the enzymatic level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the
15 same extent as if each was specifically and individually indicated to be incorporated by reference.

In a first aspect, the invention provides agents that inhibit one or more histone deacetylase isoform, but less than all specific histone deacetylase isoforms. As used herein interchangeably, the terms "histone deacetylase", "HDAC", "histone deacetylase isoform", "HDAC isoform" and
20 similar terms are intended to refer to any one of a family of enzymes that remove acetyl groups from the epsilon-amino groups of lysine residues at the N-terminus of a histone. Unless otherwise indicated by context, the term "histone" is meant to refer to any histone protein, including H1, H2A, H2B, H3, and H4, from any species. Preferred histone deacetylase isoforms
25 include class I and class II enzymes. Specific HDACs include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. By way of non-limiting example, useful agents that

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inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms, include antisense oligonucleotides and small molecule inhibitors.

The present inventors have surprisingly discovered that specific
5 inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1
10 and/or HDAC-4.

Preferred agents that inhibit HDAC-1 and/or HDAC-4 dramatically inhibit growth of human cancer cells, independent of p53 status. These agents significantly induce apoptosis in the cancer cells and cause dramatic growth arrest. They also can induce transcription of tumor suppressor
15 genes, such as p21^{WAF1}, p57^{KIP2}, GADD153 and GADD45. Finally, they exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention. By way of non-limiting example, antisense oligonucleotides and/or small molecule inhibitors of HDAC-1 and/or
20 HDAC-4 are useful for the invention.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding a specific histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In
25 other embodiments, the oligonucleotide ultimately inhibits translation of the histone deacetylase. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Preferred antisense

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oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

The antisense oligonucleotides according to the invention are complementary to a region of RNA or double-stranded DNA that encodes
5 a portion of one or more histone deacetylase isoform (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform).

For purposes of the invention, the term "complementary" means
10 having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base
15 stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

For purposes of the invention, the term "oligonucleotide" includes
20 polymers of two or more deoxyribonucleosides, ribonucleosides, or 2'-O-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of
25 the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate,

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carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleotide linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or
5 phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines, and adamantane. The term oligonucleotide also encompasses such polymers as
10 PNA and LNA. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl, or allyl group may be unsubstituted or may be substituted, *e.g.*, with
15 halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group.

Particularly preferred antisense oligonucleotides utilized in this
20 aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

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For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or
5 phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region (see *e.g.*, Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and
10 phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-O-substituted ribonucleotide region, preferably
15 comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2'-O-substituted ribonucleosides, or combinations thereof (see *e.g.*, Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and
20 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to inhibit expression of a specific histone deacetylase isoform or inhibit one or more histone deacetylase
25 isoforms, but less than all specific histone deacetylase isoforms. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding a specific histone deacetylase isoform, quantitating the amount of histone

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deacetylase isoform protein, quantitating the histone deacetylase isoform enzymatic activity, or quantitating the ability of the histone deacetylase isoform to inhibit cell growth in a an *in vitro* or *in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit
5 expression" and similar terms used herein are intended to encompass any one or more of these parameters.

Antisense oligonucleotides utilized in the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry,
10 phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*,
15 Pon, R. T., Methods in Molec. Biol. 20: 465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used to inhibit the activity of specific histone deacetylase isoforms in an
20 experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform expression according to the invention and observing any phenotypic
25 effects. In this use, the antisense oligonucleotides according to the invention is preferable to traditional "gene knockout" approaches because it is easier to use, and can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

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- Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the histone deacetylase isoform, and/or the translation of a nucleic acid molecule encoding the histone deacetylase isoform, and/or lead to the degradation of such nucleic acid. Histone deacetylase-encoding nucleic acids may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5' and 3' regions, intron-exon boundaries as well as coding sequences from a histone deacetylase family member gene. For human sequences, see *e.g.*, Yang et al., *Proc. Natl. Acad. Sci. (USA)* 93(23): 12845-12850, 1996; Furukawa et al., *Cytogenet. Cell Genet.* 73(1-2): 130-133, 1996; Yang et al., *J. Biol. Chem.* 272(44): 28001-28007, 1997; Betz et al., *Genomics* 52(2): 245-246, 1998; Taunton et al., *Science* 272(5260): 408-411, 1996; and Dangond et al., *Biochem. Biophys. Res. Commun.* 242(3): 648-652, 1998).
- Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to regions of RNA or double-stranded DNA encoding a histone deacetylase isoform (*e.g.*, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8). (see *e.g.*, GenBank Accession No. U50079 for human HDAC-1 (Fig. 1B); GenBank Accession No. U31814 for human HDAC-2; (Fig. 2B) GenBank Accession No. U75697 for human HDAC-3 (Fig. 3B; GenBank Accession No. AB006626 for human HDAC-4 (Fig. 4B); GenBank Accession No. AB011172 for human HDAC-5 (Fig. 5B); GenBank Accession No. AJ011972 for human HDAC-6 (Fig. 6B); GenBank Accession No. AF239243 for human HDAC-7 (Fig. 7B); and GenBank Accession No. AF230097 for human HDAC-8 (Fig. 8B)).

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The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Numbers X98207 (murine HDAC-1); NM_008229 (murine HDAC-2); NM_010411 (murine HDAC-3); NM_006037 (murine HDAC-4);
5 NM_010412 (murine HDAC-5); NM_010413 (murine HDAC-6); and AF207749 (murine HDAC-7)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to regions of RNA or double-stranded DNA that encode histone deacetylases from non-human animals. Antisense oligonucleotides according to these embodiments are useful as
10 tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences shown in Table I. Yet additional particularly preferred
15 oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four
20 nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar residues.

Antisense oligonucleotides used in the present study are shown in Table I.

Table 1
Sequences of Human Isotype-Specific Antisense (AS)
Oligonucleotides and Their Mismatch (MM) Oligonucleotides

Oligo	Target	Accession Number	Nucleotide Position	Sequence	Gene Position
HDAC1 AS1 HDAC1 AS2 HDAC1 MM	Human HDAC1 Human HDAC1 Human HDAC1	U50079 U50079 U50079	1585-1604 1565-1584 1585-1604	5'-GAAACGTGAGGGACTCAGCA-3' (SEQ ID NO:17) 5'-GGAAGCCAGAGCTGGAGAGG-3' (SEQ ID NO:18) 5'-GTTAGGTGAGGCACTGAGGA-3' (SEQ ID NO:19)	3'-UTR 3'-UTR 3'-UTR
HDAC2 AS HDAC2 MM	Human HDAC2 Human HDAC2	U31814 U31814	1643-1622 1643-1622	5'-GCTGAGCTGTTCTGATTTGG-3' (SEQ ID NO:20) 5'-CGTGAGCACTTCTCATTTC-3' (SEQ ID NO:21)	3'-UTR 3'-UTR
HDAC3 AS HDAC3 MM	Human HDAC3 Human HDAC3	AF039703 AF039703	1276-1295 1276-1295	5'-CGCTTTCCTTGTGATTGACA-3' (SEQ ID NO:22) 5'-GCCCTTCCTACTCATTGTGT-3' (SEQ ID NO:23)	3'-UTR 3'-UTR
HDAC4 AS1 HDAC4 MM1 HDAC4 AS2 HDAC4 MM4	Human HDAC4 Human HDAC4 Human HDAC4 Human HDAC4	AB006626 AB006626 AB006626 AB006626	514-33 514-33 7710-29 7710-29	5'-GCTGCCTGCCGTGCCACCC-3' (SEQ ID NO:24) 5'-CGTGCTGCGCTGCCACGG-3' (SEQ ID NO:25) 5'-TACAGTCCATGCAACCTCCA-3' (SEQ ID NO:26) 5'-ATCAGTCCAACCAACCTCGT-3' (SEQ ID NO:27)	5'-UTR 5'-UTR 3'-UTR 3'-UTR
HDAC5 AS	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGCTTGG-3' (SEQ ID NO:28)	3'-UTR
HDAC6 AS HDAC6 MM	Human HDAC6 Human HDAC6	AJ011972 AJ011972	3791-3810 3791-3810	5'-CAGGCTGGAATGAGCTACAG-3' (SEQ ID NO:29) 5'-GACGCTGCAATCAGGTAGAC-3' (SEQ ID NO:30)	3'-UTR 3'-UTR
HDAC7 AS	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCACA-3' (SEQ ID NO:31)	3'-UTR
HDAC8 AS1 HDAC8 AS2	Human HDAC8 Human HDAC8	AF230097 AF230097	51-70 1328-1347	5'-CTCCGGCTCCTCCATCTTCC-3' (SEQ ID NO:32) 5'-AGCCAGCTGCCACTTGATGC-3' (SEQ ID NO:33)	5'-UTR 3'-UTR

The antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents (see preparation of pharmaceutically acceptable formulations in, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the proviso that such carriers or diluents not affect their ability to modulate HDAC activity.

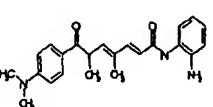
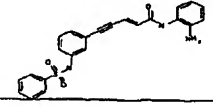
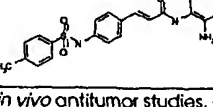
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By way of non-limiting example, the agent of the first aspect of the invention may also be a small molecule inhibitor. The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In one certain embodiment, the small molecule inhibitor is an inhibitor of one or more but less than all HDAC isoforms. By "all HDAC isoforms" is meant all proteins that specifically remove an epsilon acetyl group from an N-terminal lysine of a histone, and includes, without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8, all of which are considered "related proteins," as used herein.

Most preferably, a histone deacetylase small molecule inhibitor interacts with and reduces the activity of one or more histone deacetylase isoforms (*e.g.*, HDAC-1 and/or HDAC-4), but does not interact with or reduce the activities of all of the other histone deacetylase isoforms (*e.g.*, HDAC-2 and HDAC-6). As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of a histone deacetylase isoform that is involved in tumorigenesis.

Non-limiting examples of small molecule inhibitors useful for the invention are presented in Table 2.

Table 2

Small Molecule HDAC Inhibitors [μ M] and Their Antitumor Activities <i>In Vivo</i>												
Cpd	Inhibitor Structure	Enzyme IC ₅₀ (μ M)						% inhibitor of tumor formation in vivo				
		HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	H4-Ac	MTT	Cell Cycle Arrest EC	colon	lung	prostate
1		3	25	21	23	>50	1	3	2			
2		3	31	30	35	>30	5	4	8	53 (40,po)	54 (50,ip)	
3		3	22	45	28	>50	5	4	2	55 (40,ip)		
note: for <i>in vivo</i> antitumor studies, numbers outside brackets indicate % of inhibition of tumor growth in vivo; numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day); oral (PO) or intraperitoneal (IP) administration is indicated in brackets.												

5 The reagents according to the invention are useful as analytical tools and as therapeutic tools, including as gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

10 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell comprising contacting the cell with an agent of the first aspect of the invention. By way of non-limiting example, the agent may be an antisense oligonucleotide or a small molecule inhibitor that inhibits the expression of

15 one or more, but less than all, specific histone deacetylase isoforms in the cell.

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In one certain embodiment, the invention provides a method comprising contacting a cell with an antisense oligonucleotide that inhibits one or more but less than all histone deacetylase isoforms in the cell. Preferably, cell proliferation is inhibited in the contacted cell. Thus, the

5 antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases including benign and malignant neoplasms by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of a histone deacetylase antisense oligonucleotide

10 or a small molecule histone deacetylase inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted. Such an assessment of cell proliferation can be made by counting contacted and non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a

15 hemacytometer. Where the cells are in a solid growth (*e.g.*, a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells.

20 More preferably, the term includes a retardation of cell proliferation that is 100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, a histone deacetylase antisense oligonucleotide or a histone

25 deacetylase small molecule inhibitor that inhibits cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, to undergo growth arrest, to undergo programmed cell death (*i.e.*, to apoptose), or to undergo necrotic cell death.

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Conversely, the phrase "inducing cell proliferation" and similar terms are used to denote the requirement of the presence or enzymatic activity of a specific histone deacetylase isoform for cell proliferation in a normal (*i.e.*, non-neoplastic) cell. Hence, over-expression of a specific
5 histone deacetylase isoform that induces cell proliferation may or may not lead to increased cell proliferation; however, inhibition of a specific histone deacetylase isoform that induces cell proliferation will lead to inhibition of cell proliferation.

The cell proliferation inhibiting ability of the antisense
10 oligonucleotides according to the invention allows the synchronization of a population of a-synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene
15 and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the
20 invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection efficiency.

The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.

25 In yet other preferred embodiments, the cell contacted with a histone deacetylase antisense oligonucleotide is also contacted with a histone deacetylase small molecule inhibitor.

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In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated well known
5 pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.

10 In a particularly preferred embodiment of the invention, the antisense oligonucleotide is in operable association with a histone deacetylase small molecule inhibitor. The term "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense
15 oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule
20 inhibitor. In some preferred embodiments, an antisense oligonucleotide of the invention that targets one particular histone deacetylase isoform (*e.g.*, HDAC-1) is operably associated with a histone deacetylase small molecule inhibitor which targets the same histone deacetylase isoform. A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable
25 association is a covalent linkage between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor. Preferably, such covalent linkage is hydrolyzable by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

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In certain preferred embodiments, the covalent linkage may be directly between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone. Alternatively, the covalent linkage may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide or a lipid or a glycolipid. Other preferred operable associations include lipophilic association, such as formation of a liposome containing an antisense oligonucleotide and the histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphatidylcholine, cholesterol, phosphatidylethanolamine, and synthetic neoglycolipids, such as syallylacNAc-HDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when the antisense oligonucleotide is associated with one liposome and the small molecule inhibitor is associated with another liposome.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In one certain embodiment, the agent is an antisense oligonucleotide of the first aspect of the invention, and the method further comprises a pharmaceutically acceptable carrier. The antisense oligonucleotide and the pharmaceutically acceptable carrier are administered for a therapeutically effective period of time. Preferably, the

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animal is a mammal, particularly a domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant cell growth. Preferably, the aberrant cell growth of a neoplastic cell is increased cell growth. A neoplastic cell may be a hyperplastic cell, a cell that shows a lack of contact inhibition of growth *in vitro*, a benign tumor cell that is incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis" is used to denote the induction of cell proliferation that leads to the development of a neoplastic growth.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce neoplastic cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal, or intrarectal. When administered systemically the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.1 μM to about 10 μM . For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. One of skill in the art will appreciate that such therapeutic effect resulting in a lower effective concentration of the histone deacetylase inhibitor may vary considerably depending on the tissue, organ, or the particular animal or patient to be treated according to the invention.

In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μM to about 20 μM . In a particularly preferred embodiment, the therapeutic composition

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is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05 μM to about 15 μM . In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1 μM to about 10 μM .

5 For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred
10 embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective amount of a histone deacetylase antisense
15 oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.

 In certain preferred embodiments of the third aspect of the invention, the method further comprises administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically
20 effective period of time. In some preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

 The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a
25 sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μM to about 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule

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inhibitor from about $0.05\mu\text{M}$ to about $10\mu\text{M}$. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about $0.1\mu\text{M}$ to about $5\mu\text{M}$. For localized administration, much lower concentrations than this may be effective. Preferably, a total
5 dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred
10 embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective synergistic amount of histone deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide)
15 is about 5 mg per kg body weight per day.

Certain preferred embodiments of this aspect of the invention result in an improved inhibitory effect, thereby reducing the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*, histone
20 deacetylase small molecule inhibitor) required to obtain a given inhibitory effect as compared to those necessary when either is used individually.

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning
25 and altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example

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empirically by starting at relatively low amounts and by step-wise increments with concurrent evaluation of inhibition.

In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell

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differentiation comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In certain embodiments where the agent of the first aspect of the invention is a histone deacetylase small molecule inhibitor, therapeutic compositions of the invention comprising said small molecule inhibitor(s) are administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μ M to about

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- 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05 μM to about 10 μM . In a more preferred embodiment, the blood level of histone
- 5 deacetylase small molecule inhibitor is from about 0.1 μM to about 5 μM . For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total
- 10 dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day.
- 15 In a sixth aspect, the invention provides a method for investigating the role of a particular histone deacetylase isoform in cellular proliferation, including the proliferation of neoplastic cells. In this method, the cell type of interest is contacted with an amount of an antisense oligonucleotide that inhibits the expression of one or more specific histone deacetylase isoform,
- 20 as described for the first aspect according to the invention, resulting in inhibition of expression of the histone deacetylase isoform(s) in the cell. If the contacted cell with inhibited expression of the histone deacetylase isoform(s) also shows an inhibition in cell proliferation, then the histone deacetylase isoform(s) is required for the induction of cell proliferation. In
- 25 this scenario, if the contacted cell is a neoplastic cell, and the contacted neoplastic cell shows an inhibition of cell proliferation, then the histone deacetylase isoform whose expression was inhibited is a histone deacetylase isoform that is required for tumorigenesis. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2,

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HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

Thus, by identifying a particular histone deacetylase isoform that is
5 required for in the induction of cell proliferation, only that particular histone deacetylase isoform need be targeted with an antisense oligonucleotide to inhibit cell proliferation or induce differentiation. Consequently, a lower therapeutically effective dose of antisense oligonucleotide may be able to effectively inhibit cell proliferation.
10 Moreover, undesirable side effects of inhibiting all histone deacetylase isoforms may be avoided by specifically inhibiting the one (or more) histone deacetylase isoform(s) required for inducing cell proliferation.

As previously indicated, the agent of the first aspect includes, but is not limited to, oligonucleotides and small molecule inhibitors that inhibit
15 the activity of one or more, but less than all, HDAC isoforms. The measurement of the enzymatic activity of a histone deacetylase isoform can be achieved using known methodologies. For example, Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in
20 trichostatin A treated cells. Taunton et al. (*Science* 272: 408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous and recombinant HDAC. Both Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) and Taunton et al. (*Science* 272: 408-411, 1996) are hereby incorporated by reference.

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Preferably, the histone deacetylase small molecule inhibitor(s) of the invention that inhibits a histone deacetylase isoform that is required for induction of cell proliferation is a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of fewer
5 than all histone deacetylase isoforms.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase
10 isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6,
15 HDAC-7, or HDAC-8.

The phrase "inducing cell differentiation" and similar terms are used to denote the ability of a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or combination thereof) to induce differentiation in a contacted cell as compared to a cell that is not
20 contacted. Thus, a neoplastic cell, when contacted with a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or both) of the invention, may be induced to differentiate, resulting in the production of a daughter cell that is phylogenetically more advanced than the contacted cell.

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In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two of the reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a

5 histone deacetylase small molecule inhibitor, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain preferred embodiments, each of

10 the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

Antisense oligonucleotides that inhibit DNA methyltransferase are

15 described in Szyf and von Hofe, U.S. Patent No. 5,578,716, the entire contents of which are incorporated by reference. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.

20 In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In preferred embodiments, the cell

25 proliferation is neoplasia.

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For purposes of this aspect, it is unimportant how the specific HDAC isoform is inhibited. The present invention has provided the discovery that specific individual HDACs are involved in cell proliferation or differentiation, whereas others are not. As demonstrated in this
5 specification, this is true regardless of how the particular HDAC isoform(s) is/are inhibited.

By the term "modulating" proliferation or differentiation is meant altering by increasing or decreasing the relative amount of proliferation or differentiation when compared to a control cell not contacted with an agent
10 of the first aspect of the invention. Preferably, there is an increase or decrease of about 10% to 100%. More preferably, there is an increase or decrease of about 25% to 100%. Most preferably, there is an increase or decrease of about 50% to 100%. The term "about" is used herein to indicate a variance of as much as 20% over or below the stated numerical values.

15 In certain preferred embodiments, the histone deacetylase isoform is selected from HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1.

The following examples are intended to further illustrate certain
20 preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the
25 appended claims.

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EXAMPLES

Example 1

Synthesis and Identification of Antisense Oligonucleotides

5 Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC.
10 All oligos used were 20 base pairs in length.

 To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were
15 initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS1 and AS2 as ODNs with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base
20 mismatch.

 Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2
25 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

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Twenty-one phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3. HDAC-3
5 MM oligonucleotide was created as a control; compared to the antisense oligonucleotide, it contains a 6-base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS1 and AS2
10 were identified as ODNs with antisense activity to human HDAC-4. HDAC-4 MM1 and MM2 oligonucleotides were created as controls; compared to the antisense oligonucleotides, they each contain a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences
15 complementary to the 5' or 3' untranslated regions of the human HDAC-5 gene (GenBank Accession No. AF039691) were screened as above. HDAC-5 AS was identified as an ODN with antisense activity to human HDAC-5.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-6
20 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6 AS was identified as an ODN with antisense activity to human HDAC-6. HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

Eighteen phosphorothioate ODNs containing sequences
25 complementary to the 5' or 3' untranslated regions of the human HDAC-7 gene (GenBank Accession No. AF239243) were screened as above. HDAC-7 AS was identified as an ODN with antisense activity to human HDAC-7.

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Fourteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-8 gene (GenBank Accession No. AF230097) were screened as above. HDAC-8 AS was identified as an ODN with antisense activity to human HDAC-8.

5

Example 2 HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, human A549 cells were treated with 50 nM of antisense (AS) oligonucleotide directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or 100 nM of AS oligonucleotide directed against human HDAC-1, HDAC-2, HDAC-4, HDAC-5, HDAC-6 or HDAC-7 or the appropriate MM oligonucleotide (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

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Cells were harvested, and total RNAs were analyzed by Northern blot analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty μ g of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was performed using conventional procedures.

Figures 9A-9I present results of experiments conducted with HDAC-1 (Figure 9A), HDAC-2 (Figure 9B), HDAC-6 (Figure 9C), HDAC-3 (Figure 9D), HDAC-4 (Figures 9E and 9F), HDAC-5 (Figure 9G), HDAC-7 (Figure 9H), and HDAC-8 (Figure 9I) AS ODNs.

Treatment of cells with the respective HDAC AS ODN significantly inhibits the expression of the targeted HDAC mRNA in human A549 cells.

Example 3

HDAC OSDNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC OSDNs would inhibit HDAC protein expression, human A549 cancer cells were treated with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. OSDN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X-100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, plus protease inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 μ g of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-

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HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. *et al.*, (1999) *Mol. Cell. Biol.* 19:7816-27), and was used at a dilution of 1:1000. Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform. HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 10A, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein. Figure 10B presents dose dependent response for the inhibited expression of HDAC-1 protein in cells treated with two HDAC-1 AS ODNs. As predicted, treatment of cells with the respective mismatch (MM) control oligonucleotide does not result in a significant decrease in HDAC-1 protein expression in the treated cells.

In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

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The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are overexpressed in cancer cell lines.

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Table 3
Expression Level of HDAC Isotypes in Human
Normal and Cancer Cells

<u>States of Cell</u>	<u>Tissue Type</u>	<u>Cell Designation</u>	<u>HDAC-1</u>	<u>HDAC-2</u>	<u>HDAC-3</u>	<u>HDAC-4</u>	<u>HDAC-6</u>
Normal	Breast Epithelial	HMEC	-	+	++	+	+
Normal	Foreskin Fibroblasts	MRHF	-	+	+	++	+
Cancer	Bladder	T24	+++	++	+++	++	+++
Cancer	Lung	A549	++	+++	+++	+++	++
Cancer	Colon	SW48	+++	+++	+++	+++	+++
Cancer	Colon	HCT116	++++	+++	+++	++++	+++
Cancer	Colon	HT29	+++	+++	+++	+++	+++
Cancer	Colon	NCI-H446	++	++++	+++	++++	++
Cancer	Cervix	Hela	+++	++++	+++	+++	+++
Cancer	Prostate	DU145	+++	+++	+++	++++	+++
Cancer	Breast	MDA-MB-231	++	+++	+++	+++	++++
Cancer	Breast	MCF-7	+++	+++	+++	++	++
Cancer	Breast	T47D	+++	+++	+++	++	+++
Cancer	Kidney	293T	+++	++++	++++	++	++
Cancer	Leukemia	K562	+++	++++	++++	++++	++++
Cander	Leukemia	Jurkat T	+++	++	++++	++	++

(-): not detectable; (+): detectable; (++) : 2X over (+); (+++) : 5X over (+); (++++): 10X over (+)

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Example 4**Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis**

5 In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAMB231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

 For the apoptosis study, cells were analyzed using the Cell Death
10 Detection ELISA^{Plus} kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at
15 410 nm. The reference was set at 490 nm.

 For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a
20 hemocytometer. Percentage of inhibition was calculated as (100 - AS cell numbers/control cell numbers)%.

 Results of the study are shown in Figures 11-13, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human
25 cancer. The corresponding mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and

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treatment with human HDAC-2 OSDN has a minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells have functional p53 protein, this induction of apoptosis is independent of p53 activity.

5

Table 4
Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Growth Inhibition (AS vs. MM)

	<u>Cancer</u> <u>Cells</u>	<u>Normal</u> <u>Cells</u>		
	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	++(+)	+(+)	+/-	+/-
HDAC-2 AS	+(+)	+/-	-	+/-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	++	++	+/-
HDAC-6 AS	-	-	+/-	-

"-": no inhibition, "+": <50% inhibition, "++": 50-75% inhibition,

"+++": >75% inhibition

10

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Table 5

Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Apoptosis After 48 Hour Treatment

5

	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	+	-		-
HDAC-2 AS	-	-	-	-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	+	++	-
HDAC-6 AS	-	-	-	-
TSA (100ng/ml)	++	++	++	+

"-": ≤ 2 x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold;
"+++": 5-8X fold; "++++": 8X fold

Example 5

10 Inhibition of HDAC Isotypes Induces the Expression of Growth
Regulatory Genes

In order to understand the mechanism of growth arrest and
apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment,
15 RNase protection assays were used to analyze the mRNA expression of cell
growth regulators (p21 and *GADD45*) and proapoptotic gene *Bax*.

Briefly, human cancer A549 or T24 cells were treated with HDAC
isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total
RNAs were extracted and RNase protection assays were performed to
20 analyzed the mRNA expression level of p21 and *GADD45*. As a control,
A549 cells were treated by lipofectin with or without TSA (250 ng/ml)
treatment for 16 hours. These RNase protection assays were done

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according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San Diego, California, U.S.A.) according to the manufacturer's instructions. Protection procedures were performed using "RPA IITM Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation of the bands from autoradiograms was done by using CycloneTM Phosphor System (Packard Instruments Co. Inc., Meriden, CT). The results are shown in Figures 14, 15 and Table 6.

Table 6

Up-Regulation of p21, *GADD45* and *Bax* After Cell Treatment with Human HDAC Isotype-Specific Antisenses

	A549			T24		
	p21	<i>GADD45</i>	<i>Bax</i>	p21	<i>GADD45</i>	<i>Bax</i>
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

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Results of the experiments are presented in Table 6. The inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression. Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45* expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8 uM, 16 hrs).

Experiments were also conducted to examine the affect of HDAC antisense oligonucleotides on HDAC protein expression. In A549 cells, treatment with HDAC-4 antisene oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 15).

Example 6

Cyclin Gene Expression Is Repressed by HDAC-1 AS Treatment

Human cancer A549 cells were treated with AS1, AS2 or MM oligo directed human HDAC1 for 48 hours. Total cell lysates were harvested and analyzed by Western blot using antibodies against human HDAC1, cyclin B1, cyclin A and actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, California). AS1 or AS2 both repress expression of cyclin B1 and A. Downregulation of cyclin A and B1 expression by AS1 and AS2 correlates well with their ability to inhibit cancer cell growth. (Figure 16)

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Example 7

Inhibition of Growth in Soft Agar

5 1.3 g granulated agar (DIDFCO) was added to 100 ml deionized water and boiled in a microwave to sterilize. The boiled agar was held at 55°C until further use. Iscove's Modified Dulbecco's Medium (GIBCO/BRL), 100x Penicillin-Streptomycin-Glutamine (GIBCO/BRL) and fetal bovine serum (medicorp) were pre-warmed at 37°C. To 50 ml sterile
10 tubes was added 9 ml Iscove's medium, 2 ml fetal bovine serum and 0.2 ml 100x Pen-Strep-Gln. Then 9 ml 55°C 1.3% agar was added to each tube. The tube contents were mixed immediately, avoiding air bubbles, and 2.5 ml of the mixture was poured into each sterile 6 cm petri dish to form a polymerized bottom layer. Dishes with polymerized bottom layers were
15 then put in a CO2 incubator at 37°C until further use. In 50 ml sterile tubes were prewarmed at 37°C for each 4 cell lines/samples, 20 ml Iscove's medium, 0.4 ml 100x Pen-Strep-Gln and 8 ml fetal bovine serum. Cells were trypsinized and counted by trypan blue staining and 20,000 cells were aliquotted into a sterile 15 ml tube. To the tube was then added DMEM
20 with low glucose (GIBCO/BRL) + 10% fetal bovine serum + Pen-Strep-Gln to a final volume of 1 ml. To the prewarmed 37°C mix in the 50 ml tube was quickly added 8 ml 55°C 1.3% agar, which was then mixed well. Nine ml of this mixture was then aliquotted to each 1 ml cells in the 15 ml tube which is then mixed and 5 ml aliquotted onto the polymerized bottom
25 layer of the 6 cm culture plates and allowed to polymerize at room temperature. After polymerization, 2.5 ml bottom layer mix was gently added over the cell layer. Plates were wrapped up in foil paper and

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incubated in a CO₂ incubator at 37°C for three weeks, at which time colonies in agar are counted. The results are shown in Figure 17.

These results demonstrate that an antisense oligonucleotide complementary to HDAC-1 inhibits growth of A549 cells in soft agar, but
5 antisense oligonucleotides complementary to HDAC-2 or HDAC-6, or mismatch controls, do not.

Example 8

Inhibition of HDAC Isotypes by Small Molecules

10

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned
15 recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozier, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 96:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human
20 embryonic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium
25 precipitates, formed by mixing DNA, calcium chloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

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HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

5 For the deacetylase assay, 20,000 cpm of an [^3H]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37°C. For the HDAC-1 assay, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined
10 to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [^3H]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 4°C prior to the
15 start of the enzymatic assay. IC₅₀ values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

20

Example 9

Inhibition of HDAC Activity in Whole Cells by Small Molecules

T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were
25 extracted from the cells by standard procedures (see *e.g.* Yoshida *et al.*, *supra*) after the culture period. Twenty µg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, NY). Horse Radish
30 Peroxidase conjugated secondary antibody was used at a dilution of 1:5000

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to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

- 5 The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

Example 10

10 Inhibition of Cancer Cell Growth by HDAC Small Molecule Inhibitors

- Two thousand (2,000) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to quantitatively determine cell proliferation and cytotoxicity. Typically, HCT116 cells were plated into each well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations were added into the culture media (final DMSO concentration 1%) and incubated for 72 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO₂. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubated for overnight at 37C in incubator with 5% CO₂. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM. Optical density values were converted to cell number values by comparison to a standard growth curve for each cell line. The concentration test compound that reduces the total cell number to 50% that of the control treatment, *i.e.*, 1% DMSO, is taken as the EC₅₀ value.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

5

Example 11

Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145, 2×10^6) or human colon cancer cells (HCT116; 2×10^6) or small lung core A549 2×10^6 were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperitoneal or oral administration was initiated when the tumors reached a volume of 100 mm^3 . For intraperitoneal administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/days) were dissolved in a solution containing 65% polyethylene glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from vehicle-treated mice as controls.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

5

Example 12

Upregulation of p21 Expression and Down regulation of Cyclin Gene Expression Following Treatment with Small Molecule Inhibitor

Sulfonamide aniline (compound 3, Table 2) is a small molecule
10 HDAC1 specific inhibitor. Human HCT116 cells were treated with escalating doses of compound 3 for 16 hours. Total cell lysates were harvested and expression of p21^{WAF1}, cyclin B1, cyclin A and actin was analyzed by Western blot. Ariti-p21^{WAF1} antibody was purchased from BD Transduction Laboratories (BD Pharmingen Canada, Missasagua, Ontario).
15 Compound 3 clearly upregulates expression of p21^{WAF1} and represses the expression of cyclin A and B1. The expression profile of these cell cycle regulators correlates well with the ability of compound 3 to inhibit HCT116 proliferation in MTT assays (see Table 2),

20

Example 13

Cell Cycle Arrest Induced by HDAC Small Molecule Inhibitors

Human cancer HCT116 cells were plated at 2×10^5 per 10-cm dish and were left to attach to the dish overnight in the incubator. Cells were
25 treated with small molecule inhibitors at various concentrations (1 μ M and 10 μ M, typically, dissolved in DMSO) for 16 hours. Cells were harvested by trypsinization and washed once in 1X PBS (phosphate buffered saline). The cells were resuspended in about 200ul 1X PBS and were fixed by slowly adding 1 ml 70% ethanol at -20° C and were left at least overnight at

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-20° C. Fixed cells were centrifuged at low speed (1,000 rpm) for 5 minutes, and the cell pellets were washed again with 1X PBS. Nucleic acids from fixed cells were incubated in a staining solution (0.1% (w/v) glucose in 1X PBS containing 50 ug/ml propidium iodide) (Sigma-Aldridge, 5 Mississauga, Ontario, CA) and RNase A (final 100 units/ml, (Sigma-Aldridge, Mississauga, Ontario, CA) for at least 30 minutes in the dark at 25° C. DNA content was measured by using a fluorescence-activated cell sorter (FACS) machine. Treatment of cells with all HDAC small molecule inhibitors in Table 2 results in a significant accumulation of cancer cell in 10 G2/M phase of the cell cycle and concomitantly reduce the accumulation of cancer cells in S phase of the cell cycle. The ratio of cells in G2/M phase vs. cells in the S phase was determined. The Effective concentration (EC) of a small molecule inhibitor to induce a (G2+M)/S ratio of 2.5 is calculated, as shown in Table 2.

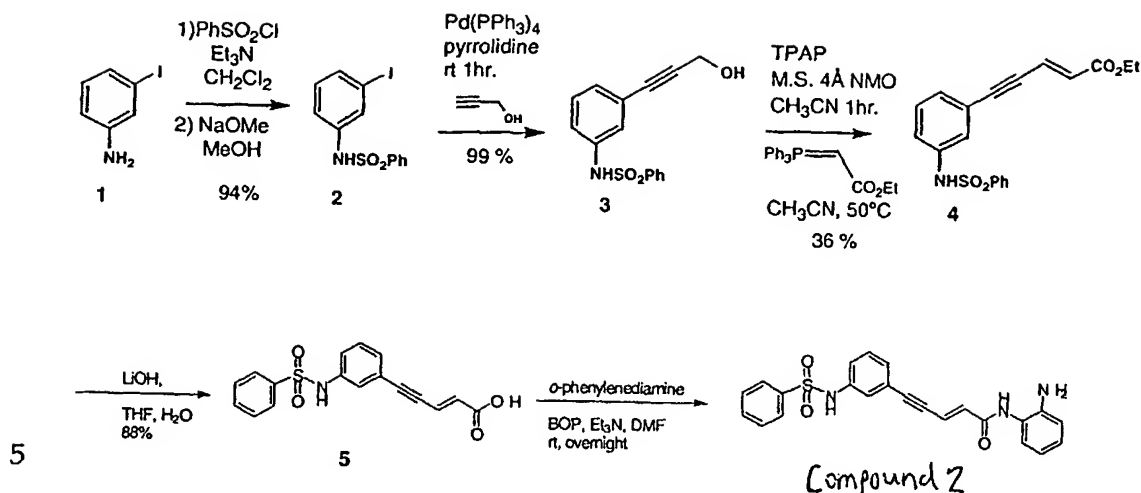
15

Example: 14

Synthesis of Small Molecule Compound No. 2

The following provides a synthesis scheme for small molecule Compound No. 2 from Table 2.

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Step 1: 3-(benzenesulfonylamino)-phenyl iodide (2)

To a solution of 3-iodoaniline (5 g, 22.8 mmol), in CH_2Cl_2 (100 mL), were added at room temperature Et_3N (6.97 mL) followed by benzenesulfonyl chloride (5.84 mL). The mixture was stirred 4 h then a white precipitate was formed. A saturated aqueous solution of NaHCO_3 was added and the phases were separated. The aqueous layer was extracted several times with CH_2Cl_2 and the combined extracts were dried over (MgSO_4) then evaporated. The crude mixture was dissolved in MeOH (100 mL) and NaOMe (6 g), was added and the mixture was heated 1 h at 60°C . The solution became clear with time and HCl (1N) was added. The solvent was evaporated under reduced pressure then the aqueous phase was extracted several times with CH_2Cl_2 . The combined organic extracts were dried over (MgSO_4) and evaporated. The crude material was purified by flash chromatography using (100% CH_2Cl_2) as solvent yielding the title compound 21 (7.68g, 94 %) as yellow solid.

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¹H NMR: (300 MHz, CDCl₃): δ 7.82-7.78 (m, 2H), 7.60-7.55 (m, 1H), 7.50-7.42 (m, 4H), 7.10-7.06 (m, 1H), 6.96 (t, J = 8Hz, 1H), 6.87 (broad s, 1H).

Step 2: 3-(benzenesulfonylamino)-phenyl-propargylic alcohol (3)

To a solution of 2 (500 mg, 1.39 mmol) in pyrrolidine (5 mL) at room temperature was added Pd(PPh₃)₄ (80 mg, 0.069 mmol), followed by CuI (26 mg, 0.139 mmol). The mixture was stirred until complete dissolution. Propargylic alcohol (162 •L, 2.78 mmol) was added and stirred 6 h at room temperature. Then the solution was treated with a saturated aqueous solution of NH₄Cl and extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The residue was purified by flash chromatography using hexane/AcOEt (1:1) as solvent mixture yielding 3 (395 mg, 99 %) as yellow solid.

¹H NMR: (300 MHz, CDCl₃): δ 7.79-7.76 (m, 2H), 7.55-7.52 (m, 1H), 7.45 (t, J = 8Hz, 2H), 7.19-7.15 (m, 3H), 7.07-7.03 (m, 1H), 4.47 (s, 2H).

Step 3: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoate (4)

To a solution of 3 (2.75 g, 9.58 mmol) in CH₃CN (150 mL) at room temperature were added 4-methylmorpholine N-oxide (NMO, 1.68 g, 14.37 mmol) followed by tetrapropylammonium perruthenate (TPAP, 336 mg, .958 mmol). The mixture was stirred at room temperature 3 h, and then filtrated through a Celite pad with a fritted glass funnel. To the filtrate carbethoxymethylenetriphenyl-phosphorane (6.66 g, 19.16 mmol) was added and the resulting solution was stirred 3 h at room temperature. The solvent was evaporated and the residue was dissolved in CH₂Cl₂ and washed with a saturated aqueous solution of NH₄Cl. The aqueous layer was extracted several times with CH₂Cl₂, then the combined organic extract were dried over (MgSO₄) and evaporated. The crude material was purified

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by flash chromatography using hexane/AcOEt (1:1) as solvent mixture giving 4 (1.21 g, 36%) as yellow oil.

¹H NMR: (300 MHz, CDCl₃): δ 7.81 (d, J = 8 Hz, 2H), 7.56-7.43 (m, 3H), 7.26-7.21 (m, 3H), 7.13-7.11 (m, 1H), 6.93 (d, J = 16 Hz, 1H), 6.29 (d, J = 16 Hz, 1H), 4.24 (q, J = 7 Hz, 2H), 1.31 (t, J = 7 Hz, 3H).

Step 4: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenic acid (5)

To a solution of 4 (888 mg, 2.50 mmol) in a solvent mixture of THF (10 mL) and water (10 mL) at room temperature was added LiOH (1.04 g, 25.01 mmol). The resulting mixture was heated 2 h at 60 °C and treated with HCl (1N) until pH 2. The phases were separated and the aqueous layer was extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The crude residue was purified by flash chromatography using CH₂Cl₂/MeOH (9:1) as solvent mixture yielding 5 (712 mg, 88 %), as white solid.

¹H NMR: (300 MHz, DMSO-*d*₆): δ 7.78-7.76 (m, 2H), 7.75-7.53 (m, 3H), 7.33-7.27 (m, 1H), 7.19-7.16 (m, 3H), 6.89 (d, J = 16 Hz, 1H), 6.33 (d, J = 16 Hz, 1H).

Step 5: Compound 2

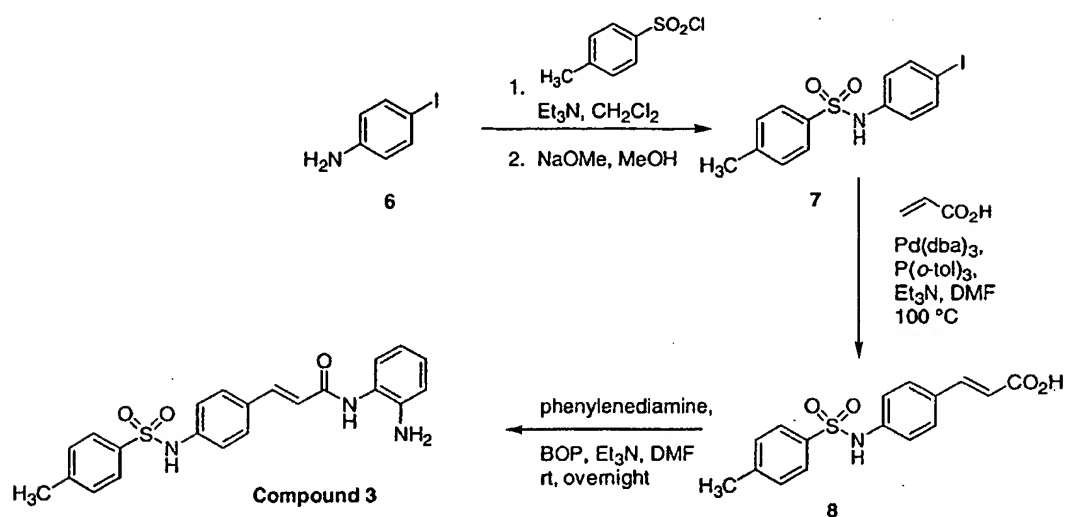
Coupling of 5 with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) afforded the anilide **Compound 2**.

¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7 Hz); 7.35 (d, 1H, J=6.9 Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9 Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 15
Synthesis of Small Molecule Compound No. 3

- 5 The following provides a synthesis scheme for Compound No. 3 from Table 2.



10 Step 1: 3-[4-(toluenesulfonylamino)-phenyl]-2-propenoic acid (8)

- To a solution of **7** (1.39 mmol), in DMF (10 mL) at room temperature were added tris(dibenzylideneacetone)dipalladium(0) ($\text{Pd}(\text{dba})_3$; 1.67 mmol), tri-*o*-tolylphosphine ($\text{P}(o\text{-tol})_3$, 0.83 mmol), Et_3N (3.48 mmol) and finally acrylic acid (1.67 mmol). The resulting solution was degassed and
- 15 purged several times with N_2 , then heated overnight at 100°C . The solution was filtrated through a Celite pad with a fritted glass funnel then the filtrate was evaporated. The residue was purified by flash chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5) as solvent mixture yielding the title compound **8**.

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Step 2: N-Hydroxy-3-[4-(benzenesulfonylamino)-phenyl]-2-propenamide(Compound 3)

The acid 8 was coupled with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium

- 5 hexafluorophosphate (BOP) to afford the anilide **Compound 3**.

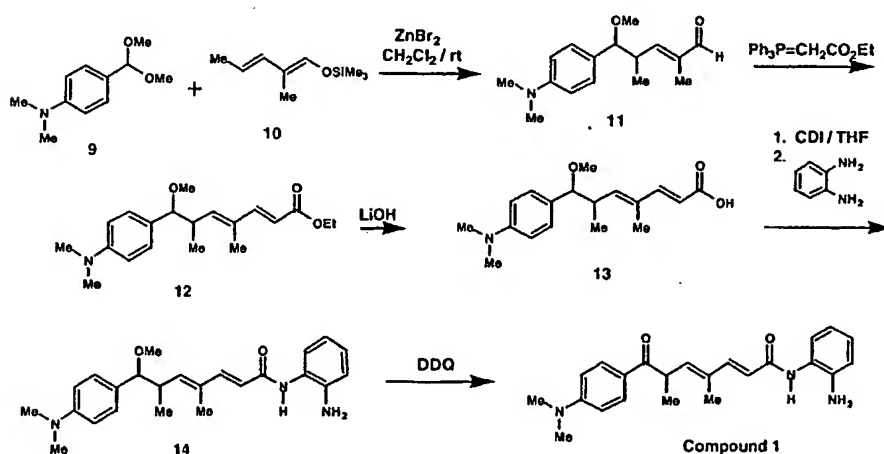
¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

- 10 ¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 16

Synthesis of Small Molecule No. Compound 1

- 15 The following provides a synthesis scheme for small molecule Compound No. 1 from Table 2.



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Step 1: (11)

To a stirred solution of *p*-anisaldehyde dimethyl acetal (9) (10 mmol) in dry CH_2Cl_2 (60 mL) at rt was added 2-methyl-1-trimethylsilyloxypenta-1,3-diene (10) (*Tetrahedron*, 39: 881 (1983)) (10 mmol) followed by catalytic amount of anhydrous ZnBr_2 (25 mg). After being stirred for 5 h at rt, the reaction was quenched with water (20 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (2 \times 25 mL). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure.

Purification of the crude product by flash silica gel chromatography (25% ethyl acetate in hexane) afforded the desired aldehyde 11 in 68% yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) δ 9.29 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.29 (dq, J = 9.9, 1.2 Hz, 1H), 3.96 (d, J = 6.6 Hz, 1H), 3.20 (s, 3H), 3.05 (m, 1H), 2.94 (s, 6H), 1.60 (d, J = 0.9 Hz, 3H), 1.12 (d, J = 6.9 Hz, 3H).

Step 2: (12)

A mixture of aldehyde 11 (5.14 mmol) and ethyl (triphenylphosphoranylidene)acetate (2.15 g, 6.16 mmol) in toluene (25 mL) was heated at reflux overnight under N_2 . After removal of the solvent under reduced pressure, the crude product obtained was purified by flash silica gel chromatography (10% ethyl acetate in hexane) to give the title compound 12 in 96 % yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) δ 7.21 (dd, J = 15.6, 0.9 Hz, 1H), 7.06 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.69 (d, J = 15.6 Hz, 1H), 5.67 (br. d, J = 9.0 Hz, 1H), 4.17 (q, J = 7.2 Hz, 2H), 3.87 (d, J = 6.9 Hz, 1H), 3.18 (s, 3H), 2.93 (s, 6H), 2.81 (m, 1H), 1.59 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.05 (d, 6.6 Hz, 3H).

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Step 3: (13)

To a stirred solution of diene ester **12** (1.24 mmol) in methanol (10 mL) at rt was added aqueous LiOH 0.5 N solution (1.7mmol). After being stirred at 40 °C for 16 h, methanol was removed under reduced
5 pressure and the resulting aqueous solution was acidified with 3N HCl (pH = ca. 4), extracted with ethyl acetate (25 × 3 mL), dried (MgSO₄), and concentrated under reduced pressure to give the desired carboxylic acid **13** in 98 % yield: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.21 (d, J = 15.6, 0.6 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.61 (d, J =
10 15.6 Hz, 1H), 5.60 (br. d, J = 10.0 Hz, 1H), 3.85 (d, J = 7.5 Hz, 1H), 3.13 (s, 3H), 2.87 (s, 6H), 2.81 (m, 1H), 1.52 (d, J = 1.5 Hz, 3H), 1.06 (d, J = 6.6 Hz, 3H).

Step 4: (14)

To a solution of carboxylic acid **13** (0.753 mmol) in anhydrous
15 THF (10 mL) was added 1,1'-carbonyldiimidazole (0.790 mmol) at rt, and the mixture was stirred overnight. To the resulting solution was added 1,2-phenylenediamine (5.27 mmol), followed by trifluoroacetic acid (52 µl), and the reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with ethyl acetate (30 mL), washed with saturated NaHCO₃
20 solution (5 mL) and then water (10 mL), dried (MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in toluene) afforded the title compound **14** in 61% yield, as a mixture of two isomers in a ca.3 : 1 ratio: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.28-7.02 (m, 5H), 6.79 (m, 2H), 6.68 (d, J = 8.7 Hz, 2H), 5.83 (d, J = 15.0 Hz,
25 1H), 5.69 (d, J = 9.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 1H), 3.19 (s, 3H), 2.94 (s, 6H), 2.80 (m, 1H), 1.61 (br. s, 3H), 1.07 (d, J = 6.6 Hz, 3H).

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Step 5: (Compound 1)

To a stirred solution of compound 14 (0.216 mmol) in wet benzene (2 mL, benzene : H₂O = 9 : 1) at room temperature was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.432 mmol). After being stirred
5 vigorously for 15 min., the mixture was diluted with ethyl acetate (30 mL), washed with water (2 × 5 mL), dried (anhydr. MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in hexanes, and then ethyl acetate only) afforded the title compound 35 (6 mg, 7% yield): ¹H NMR (300 MHz, CDCl₃) δ 7.83 (d, J = 9.0, 2H), 7.87 (br. s, 1H),
10 7.29 (d, J = 15.6 Hz, 1H), 7.27 (d, 7.8 Hz, 1H), 7.00 (m, 1H), 6.72 (m, 2H), 6.62 (d, J = 9.0 Hz, 2H), 5.97 (d, J = 15.6 Hz, 1H), 5.97 (d, J = 9.3 Hz, 1H), 4.34 (dq, J = 9.3, 6.9 Hz, 1H), 3.03 (s, 3H), 1.87 (br. s, 3H), 1.29 (d, J = 6.9 Hz, 3H); ¹³C
NMR (75 MHz, CDCl₃)
δ 12.6, 17.6, 39.9, 40.8, 110.7, 118.0, 119.0, 119.3, 123.8, 124.4, 125.1, 126.9,
15 130.6, 132.5, 140.8, 146.2, 153.4, 164.8, 198.6.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific
20 embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. An agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms.
- 5 2. The agent according to claim 1, wherein the agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, is an oligonucleotide.
3. The oligonucleotide according to claim 2, wherein the
10 oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms.
4. The oligonucleotide according to claim 3, wherein the oligonucleotide is a chimeric oligonucleotide.
- 15 5. The oligonucleotide according to claim 3, wherein the oligonucleotide is a hybrid oligonucleotide.
6. The oligonucleotide according to claim 3, wherein the
20 oligonucleotide is complementary to a region of RNA or double-stranded DNA selected from the group consisting of
 - (a) a nucleic acid molecule encoding a portion of HDAC-1 (SEQ ID NO:2),
 - (b) a nucleic acid molecule encoding a portion of HDAC-2 (SEQ
25 ID NO:4),

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- (c) a nucleic acid molecule encoding a portion of HDAC-3 (SEQ ID NO:6),
- (d) a nucleic acid molecule encoding a portion of HDAC-4 (SEQ ID NO:8),
- 5 (e) a nucleic acid molecule encoding a portion of HDAC-5 (SEQ ID NO:10),
- (f) a nucleic acid molecule encoding a portion of HDAC-6 (SEQ ID NO:12),
- (g) a nucleic acid molecule encoding a portion of HDAC-7 (SEQ ID NO:14), and
- 10 (h) a nucleic acid molecule encoding a portion of HDAC-8 (SEQ ID NO:18).

7. The oligonucleotide according to claim 6 having a nucleotide
15 sequence of from about 13 to about 35 nucleotides.

8. The oligonucleotide according to claim 6 having a nucleotide
sequence of from about 15 to about 26 nucleotides.

20 9. The oligonucleotide according to claim 6 having one or more
phosphorothioate internucleoside linkage, being 20-26 nucleotides in
length, and being modified such that the terminal four nucleotides at the 5'
end of the oligonucleotide and the terminal four nucleotides at the 3' end of
the oligonucleotide each have 2'-O- methyl groups attached to their sugar
25 residues.

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10. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-1 (SEQ ID NO:2).
- 5 11. The oligonucleotide according to claim 10 that is SEQ ID NO:17 or SEQ ID NO:18.
12. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded
10 DNA encoding a portion of HDAC-2 (SEQ ID NO:4).
13. The oligonucleotide according to claim 12 that is SEQ ID NO:20.
- 15 14. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-3 (SEQ ID NO:6).
15. The oligonucleotide according to claim 14 that is SEQ ID
20 NO:22.
16. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-4 (SEQ ID NO:8).
- 25

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24. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-8 (SEQ ID NO:16).

5 25. The oligonucleotide according to claim 24 that is SEQ ID NO:32 or SEQ ID NO:33.

26. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the agent according to
10 claim 1.

27. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the oligonucleotide according to claim 3.
15

28. The method according to claim 27, wherein cell proliferation is inhibited in the contacted cell.

29. The method according to claim 27, wherein the
20 oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth retardation.

30. The method according to claim 27, wherein the
25 oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth arrest.

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31. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo programmed cell death.

5 32. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo necrotic cell death.

33. The method according to claim 27, further comprising
10 contacting the cell with a histone deacetylase small molecule inhibitor.

34. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
15 agent of claim 1.

35. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
20 oligonucleotide of claim 3.

36. The method according to claim 35, wherein the animal is a human.

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37. The method according to claim 35, further comprising administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

5

38. A method for identifying a histone deacetylase isoform that is required for the induction of cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in the induction of cell proliferation indicates that the histone deacetylase isoform is required for the induction of cell proliferation.

10

39. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

15

40. A method for identifying a histone deacetylase isoform that is required for cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in cell proliferation indicates that the histone deacetylase isoform is required for cell proliferation.

20

41. The method according to claim 40, wherein the inhibitory agent is an oligonucleotide of claim 3.

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42. A method for identifying a histone deacetylase isoform that is required for the induction of cell differentiation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein an induction of cell differentiation indicates that the histone
5 deacetylase isoform is required for the induction of cell proliferation.

43. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

10 44. A method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide
15 that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor.

45. A method for modulating cell proliferation or differentiation of a cell comprising inhibiting a specific HDAC isoform that is involved in
20 cell proliferation or differentiation by contacting the cell with an agent of claim 1.

46. The method according to claim 45, wherein the cell proliferation is neoplasia.

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47. The method according to claim 46, wherein the histone deacetylase isoform is selected from the group consisting of HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8.

5 48. The method according to claim 47, wherein the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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MAQTQGTTRRKVCYYYDGVGNYYYQGHPMKPHRIRMTNLLN
YGLYRKMEIYRPHKANAEMTKYHSDDDYIKFLRSIRPDNMSEYSKMQRFNVGEDCPV
FDGLFEFCQLSTGGSVASAVKLNKQTDIAVNWAGGLHHAKKSEASGFCYVNDIVLAI
LELLKYHQRVLYIDIDIHHGDDGVEEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGK
GKYYAVYPLRDGIDDESIEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNL
TIKGHAKCVEFVKSFNLPMLMLGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYF
EYFGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEE
SGDEDEDDPKRISICSSDKRIACEEEFSDSEEEGEGGRKNSSNFKKAKRVKTEDEKE
KDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA (SEQ ID NO:1)

FIG. 1A

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1 atgtctgggg tctctgcccg ctggtgctgc tgtctcccac tgggtcatcc tgagaacaca
 61 gcctgagcgr ctctgtcact cggggtagac cagcgggga cgcgagcaag atggcgacaga
 121 cgcagggcac ccggaggaaa gtctgttact actacgacgg gaatcggcat gactcataat ttgctgctca
 181 atggacaagg ccaccaatg ctaccgaaa cactacatta aattcttgcg tcaacgttgg tgaggactgt ccagtattcg
 241 actatggtct tgaccaagta cagcaagcag atgcagagat cagttgtcta ctggtggttc attggcctgc catgcaaaaga
 301 tgaccaagta cagcaagcag atgcagagat cagttgtcta ctggtggttc attggcctgc catgcaaaaga
 361 tgtcggagta atggttctgt gcagacggac atctggcttc tgttacgtca ctgtacattg gaccgggtca tgaactgctc
 421 atggtcctgt taaataagca gtagcggag atctggcttc tgttacgtca ctgtacattg gaccgggtca tgaactgctc
 481 taaataagca gtagcggag atctggcttc tgttacgtca ctgtacattg gaccgggtca tgaactgctc
 541 agtccgaggc taaagtatca ctagaggggtg ctacaccag aactggggac gctccgagac agtaatggag tggggatcgg
 601 taaagtatca ctagaggggtg ctacaccag aactggggac gctccgagac agtaatggag tggggatcgg
 661 aagagggcctt acttcccagg gctccgagac agtaatggag tggggatcgg tgtcaagagc cgttgcccgg gcttccatc
 721 acttcccagg gctccgagac agtaatggag tggggatcgg tgtcaagagc cgttgcccgg gcttccatc
 781 taaactaccc tcatgtccaa actccctatc gtgtggaatt ccatcgttaa tccctaataga acatcagttcc
 841 tcatgtccaa actccctatc gtgtggaatt ccatcgttaa tccctaataga acatcagttcc
 901 actccctatc gtgtggaatt ccatcgttaa tccctaataga acatcagttcc
 961 gtgtggaatt ccatcgttaa tccctaataga acatcagttcc
 1021 ccatcgttaa tccctaataga acatcagttcc
 1081 tccctaataga acatcagttcc
 1141 acatcagttcc tccctaataga acatcagttcc
 1201 agcagactgtt tccctgagga cgcactcctt tgagaaacctt gaggagagtg gacaaacgaa ttgctgtga
 1261 tccctgagga cgcactcctt tgagaaacctt gaggagagtg gacaaacgaa ttgctgtga
 1321 gcatctcgat ctgctcctct agaggggggc tgaaaaagag gaagacccag aggagaagaa gtgtcaagga gggccctgaa
 1381 aagagggaggg agaggggggc tgaaaaagag gaagacccag aggagaagaa gtgtcaagga gggccctgaa
 1441 aaacagagga ggagaaagga cagctctggc
 1501 aaacaaagga ggagaaagga cagctctggc
 1561 tggacctctc cagctctggc

c (SEQ ID NO:2)

FIG. 1B

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MAYSQGGKKCKVCYYYDGDIGNYYYGQHPMKPHRIRMTHNLLL
NYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMHIPFNVGEDCP
AFDGLFEFCQLSTGGSVAGAVKLNRRQQTDMAVNWAGGLHHAKKYEASGFCYVNDIVLA
ILELLKYHQRVLYIDIDIHHRGDGVEEAFYTTDRVMTVSFYGEYFFPGTGLRDIGAG
KGKYYAVNFPKCDGIDDESYGQIFKPIISKVMEYQPSAVVLQCGADSLSGDRLGCFN
LTVKGHAKCVEVVKTFNLP LLMLGGGYTILRNVARCWTYETAVALDCEIPNELPYNDY
FEYFGPDFKLHISPSNMTNQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHE
DSGDEGEDPDKRISIRASDKRIACDEEFSDEGEGRNVADHKKGAKARIEED
KKETEDKKTVDVKEEDKSKDNSGEKTDTKGKSEQLSNP (SEQ ID NO:3)

FIG. 2A

1 cgccgagctt tggcacctc tggcgggtgg taccgagcct tccggcgcc cctcctctc
 61 ctcccacgg cctgcccctc ccgcggggac tatcgcccc agcagcagca tccggcgcc acgtttccct cagcccttt
 121 ctctccggc cgagccgcg ggcagcagc agcagcagca taccagtgac gaggcaggg gaggcaggg gaggcaggg
 181 gtggcgggc acgacggtga tctggaat tatgttaatt gaagaatga gataacatga ctgagtatg taagcagatg
 241 tgcctactc cctcatagaa tccgcatgac ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg
 301 cctcatagaa tccgcatgac ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg atattgtgct
 361 gaaatatata tatatacaat ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 421 tatatacaat ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata cgtgtaata
 481 catataatga ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 541 ctctcaactg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 601 gctgttaatt ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 661 tacgttaatt ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 721 tatatcgata ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 781 cgtgtaata ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 841 agggataatg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 901 atagacgatg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 961 tatcaacctt ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1021 ggtgtttcca ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1081 aacttaccat ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1141 tggacatatg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1201 gattactttg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1261 aaccagaaca ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1321 atgttacctc ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1381 gacagtggag ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1441 aagcggatag ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1501 agaaatgtgg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1561 gaaacagagg ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1621 gaaaaaacag ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1681 tctaccaaat ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1741 gaagacttct ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1801 actttttcgt ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1861 aaattttctt ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1921 gtcaaaaaaa ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata
 1981 aaaag (SEQ ID NO:4) ggcacctaa ttctacggtc atgttgaga gctgttcagt gggctggagg tagatatcca cgtgttaata

FIG. 2B

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MAKTVAIFYDPDVGNFHYGAGHPMKPHRLALTHSLVLHYGLYKK
MIVFKPYQASQHDMCRFHSEYIDFLQRVSPTNMQGFTKSLNAPNVGDDCPVFPGLFE
FCsRYTGASLQATQLNNKICDIANWAGGLHHAKKFEASGFCYVNDIVIGILELLLY
HPRVLYIDIDIHHGDGVQEAFFYLTDRVMTVSFHKYGNYYFFPGTGDMEVGAESGRYYC
LNVPLRDGIDDDQSYKHLFQPVINQVVDYQPTCIVLQCGADSLGCDRLGCFNLSIRGH
CECVEYVKSFNIPPLLVLGGGYTVRNVARCWTYETSLVVEEAISEELPYSEYFEYFAP
DFTLHPDVSTRIENQSRQYLDQIRQTIFENLKMNLNHAPSVQIHDPADLLTYDRTDE
ADAEERGPEENYSRPEAPNEFYDGDHDNDKESDVEI (SEQ ID NO:5)

FIG. 3A

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1 ggaattcgcg gccgcggcgg gccctatttct caagaccctg caagacccga acgaccccca gcgcgggagg tgcggggcct gctccgcgcg gcaccatggc
 61 ccctatgaag ccccatcgcc cccatcgcc acgaccccca tggcatcgac agccatcgac ccatagccctg cctccgcgcg tccactacg gagctggaca
 121 taagaagatg atcgctctca acatcgctca acgttagcga gcgcctggtg acatggtgat tggcatcgctg gcaaggagca tccactacg tccactacg
 181 ctccgaggac tacattgact tccatcgact acgttagcga gcgcctggtg acatggtgat tggcatcgctg gcaaggagca tccactacg tccactacg
 241 caagagtcct aatgccttca acgttagcga gcgcctggtg acatggtgat tggcatcgctg gcaaggagca tccactacg tccactacg
 301 gttctgctcg cgttacacag gccattaacb tatgtcaacg tacattgaca cgggtgcctt tggcatcgctg gcaaggagca tccactacg tccactacg
 361 ctgtgatatb tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 421 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 481 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 541 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 601 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 661 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 721 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 781 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 841 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 901 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 961 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1021 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1081 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1141 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1201 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1261 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1321 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1381 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1441 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1501 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1561 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1621 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1681 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1741 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1801 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1861 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc
 1921 tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc tggcttctgc

FIG. 3B

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MLAMKHQQELLEHQKLERHRQEQELEKQHREQKLQQLKNKEKG
KESAVASTEVMKMLQEFVLNKKKALAHPNLNHCISSCPXYWYKTOHSSLDQSSPPQS
GVSTSYNHPVLGMYDAKDDFPLRKTASEP NLKLSRLKQKVAERRSSPLLRRKDGPPV
TALKKRPLDVTDSACSSAPGSGPSSPNNSSGSVAENGIAPAVPSIPAETSLAHLVA
REGSAAPLPLYTSPSLPNITLGLPATGPSAGTAGQQDTERLTLPALQQRLSLFFPGTHL
TPYLTSPLERDGGAAHSPLLQHMVLLLEQPPAQAPLVTGLGALPLHAQSLVGADRVSP
SIHKLRRQHRPLGRTQSAPLPQNAQALQHLVIOQQHQQFLEKHKQQFQQQLQMNKIIP
KPSEPARQPESHPEETEEELREHQALLDEPYLDRLPQGQKEAHAQAQGVVKQEPYESDE
EEAEPREVEPGQRPSEQELLFRQQAALLLEQQRIHQRLRNYQASMEAAGIPVSFGGHR
PLSRAQSSPASATFPVSVQEPPTKPRFTTGLVYDTLMLKHQCTCGSSSSHPEHAGRIQ
SIWSRLQETGLRGKCECIRGRKATLEELQTVHSEAHTLLYG TNPLNRQKLD SKKLLGS
LASV FVRLPCGGVGVDSDTIWNEVHSAGAAARLAVGCVVELVFKVATGELKNGFAVVRP
PGHHAESTPMGFCYFNSVAVAAKLLQQLSVSKILLIVDWDVHHGNGTQQA FYSDPSV
LYMSLHRYDDGNFFPGSGAPDEVGTGPGVGFNVNMAFTGGLDPPMGDAEYLA AFRTVV
MPIASEFAPDVVLASSGFD AVEGHPTPLGGYNLSARCFGYLT KQLMGLAGGRIVL ALE
GGHDLTAICDASEACVSALLGNELDPLPEKVLQQRPNANAVRSM EKVMEIHSKYWRCL
QRTTSTAGRSLIEAQT CENEEAETVTAMASLSVG VKPAEKRPDEEPMEEEPPL (SEQ ID NO: 7)

FIG. 4A

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1  ggaggttgtg  gggccgccgc  cgggagcac  cgtccccgcc  gccgccgag  cccgagcccg
61  agcccgcgca  ccgcccgcg  ccgcccgcg  cgcccgccga  acagcctccc  agcctgggcc
121  ccggcgggcg  ccgtggccgc  gtcccggctg  tcgcccggccg  agcccagacc  cgcgcgccgg
181  cgggtggcgg  cgcaggctga  ggagatgcgg  cgcggagcgc  cggagcaggg  ctagagccgg
241  ccgcccgcgc  ccgcccgcgt  aagcgcagcc  ccggcccggc  gccgcgggc  catgtccgc
301  cggtccacac  cgccccgcg  cagcctgcag  gccttggagc  ccgcccgggc  tggacgccgc
361  cggtccacac  cgccccgcg  cgcccccgcg  ggagcggggg  gccagcgcctg  gccgcgccgc
421  gtgggacccc  ccggtcccca  cgcggccggg  gggccctctg  gacctttcca  cccgcgccgc
491  gaggcggcct  cgcccgcgg  ggccggggcg  cgggggtggg  caccggcagcc  agcggcgccg
541  tctcccggcg  cgggggccgc  gccccccgag  caggttcatc  tgcagaagcc  agcggacgcc
601  tctgttcaac  ttgtgggcta  cccagccctc  accgtcccg  tacttgtatg  cggcgcctga
661  acgtctgtga  cccagccctc  tatcgtttcc  gtggaaattt  tgagccattt  agtttggagc
721  tcgttggagc  caatgagctc  caaagccat  ccagatggac  ttcttggccg  aaaggagtgg
781  acattgctag  tgaatcccg  ccgctgaac  cccatgcccc  gacgggtgga  agaccagcca
841  gtggagctgc  tgcaagtggc  cccccggca  gegcccatgg  gccgcgcct  tgtggccacg
901  gcgctgcctc  ctgtggcaga  gccggccctg  cgggagcagc  acccgccct  ggaccaccag
961  tctcactgc  ctgtggcaga  gccggccctg  cgggagcagc  acccgccct  ggagctcctg

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FIG. 4B-1

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1021 gcgctcaagc agaagcagca gatccagagg cagatcctca tgcgcgagtt ccagaggcag
 1081 cacgagcagc tctcccgga ccatgaaagc agcaggagct agcaggagc agcaggagc
 1141 gagatgctgg ccatgaaagc agcaggagct agcaggagc agcaggagc agcaggagc
 1201 caccgcccag aacaaggaga gaatttgtcc agagaccctc cccagagcgg gatgacttcc
 1261 aacaaggaga gaatttgtcc agagaccctc cccagagcgg gatgacttcc gatgacttcc
 1321 gaatttgtcc agagaccctc cccagagcgg gatgacttcc gatgacttcc gatgacttcc
 1381 agagaccctc cccagagcgg gatgacttcc gatgacttcc gatgacttcc gatgacttcc
 1441 cccagagcgg gatgacttcc gatgacttcc gatgacttcc gatgacttcc gatgacttcc
 1501 gatgacttcc gatgacttcc gatgacttcc gatgacttcc gatgacttcc gatgacttcc
 1561 aagcagaaag gtcactgctc ggctccggac gcgcccgcgg gaaggctcgg
 1621 gtcactgctc ggctccggac gcgcccgcgg gaaggctcgg gaaggctcgg
 1681 ggctccggac gcgcccgcgg gaaggctcgg gaaggctcgg gaaggctcgg
 1741 gcgcccgcgg gaaggctcgg gaaggctcgg gaaggctcgg gaaggctcgg
 1801 gaaggctcgg gaaggctcgg gaaggctcgg gaaggctcgg gaaggctcgg
 1861 ggctccggac gcgcccgcgg gaaggctcgg gaaggctcgg gaaggctcgg
 1921 acccttcccg ctgagcacct atggtctctac cccctccacg cggcagcacc
 1981 ctgagcacct atggtctctac cccctccacg cggcagcacc cggcagcacc
 2041 atggtctctac cccctccacg cggcagcacc cggcagcacc cggcagcacc
 2101 cccctccacg cggcagcacc cggcagcacc cggcagcacc cggcagcacc
 2161 cggcagcacc cggcagcacc cggcagcacc cggcagcacc cggcagcacc
 2221 ctgagcacct atggtctctac cccctccacg cggcagcacc cggcagcacc
 2281 tccagcagc atggtctctac cccctccacg cggcagcacc cggcagcacc
 2341 cagccggaga gacgagccct cagggtgaagc agccggggcc ctggagcagc
 2401 gacgagccct cagggtgaagc agccggggcc ctggagcagc ctggagcagc
 2461 cagggtgaagc agccggggcc ctggagcagc ctggagcagc ctggagcagc
 2521 agccggggcc ctggagcagc ctggagcagc ctggagcagc ctggagcagc
 2581 ctggagcagc ctggagcagc ctggagcagc ctggagcagc ctggagcagc
 2641 atccccgtgt gccaccttcc gtgtatgaca gaggacgccc gaggacgccc
 2701 gccaccttcc gtgtatgaca gaggacgccc gaggacgccc gaggacgccc
 2761 gtgtatgaca gaggacgccc gaggacgccc gaggacgccc gaggacgccc
 2821 gaggacgccc gaggacgccc gaggacgccc gaggacgccc gaggacgccc

FIG. 4B-2

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2881 aaatgcgagt gcatccgcgg acgcaaggcc accctggaag agctacagac ggtgcactcg
 2941 gaagcccaca cctccttgta tggcacgaac cccctcaacc ggcagaaact ggacagtaag
 3001 aaacttctag gctcgtctcg ctcctgttcc gtccggctcc ctgcggtgg ggttggggtg
 3061 gacagtgaca ccatatggaa cgaggtgcac cgaggtggcc acaggggagc tgaaaaatgg ctttgctgtg
 3121 tgcgtggtag agctggctct ctggacacca tgcggaggag gacacgcca agcaggttgg ctacttcaac
 3181 gtccgccccc tccgcggccc tggcagccaa gcttctgcag gcttctacag cgtcctcatc
 3241 tccgcggccc gtggactggg acgtgcacca tggaaacggg ccgctacgac acccagcagg
 3301 gtggactggg gtccctgtaca tgtccctcca tgggcacagg gcccgcggtg gatgggaact
 3361 gtccctgtaca tgtccctcca tgggcacagg gcccgcggtg gatgggaact tcttccagg
 3421 cctgatgagg tggcctggacc ccccatggg agacgctgag gcttggcgg taacttcaacg
 3481 ggcctggacc ccccatggg agacgctgag gcttggcgg taacttggcgg tcaacacggc
 3541 ccgatcgcca gagggtccac tgcgtggcct cccgatgtg cccgatgtg gcttcaagg
 3601 gagggtccac tgcgtggcct cccgatgtg cccgatgtg gcttcaagg gcttcaagg
 3661 acgaagcagc gacctgacct cctgatcctc atggagaaag acagcggggc
 3721 gacctgacct cctgatcctc atggagaaag acagcggggc acgcccattg cccatgggag
 3781 cctgatcctc atggagaaag acagcggggc acgcccattg cctcgtctgtc aggaagcctc
 3841 atggagaaag acagcggggc acgcccattg cctcgtctgtc aggaagcctc tgaagctca
 3901 acagcggggc acgcccattg cctcgtctgtc aggaagcctc tgaagctca tgaagctca
 3961 acgcccattg cctcgtctgtc aggaagcctc tgaagctca tgaagctca tgaagctca
 4021 cccatgggag tgtctctgtc gggctctctc gggctctctc cagagcctc
 4081 tgtctctgtc gggctctctc gggctctctc cagagcctc agatgaggag ccttctctgtc
 4141 gggctctctc gggctctctc gggctctctc cagagcctc agatgaggag ccttctctgtc
 4201 cggccaggcc cagagcctc gggctctctc gggctctctc cagagcctc agatgaggag
 4261 aacacgggac agacgcccgc cagagcctc gggctctctc gggctctctc cagagcctc
 4321 tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc
 4381 tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc tggcgggtcc
 4441 caaacttgat taaacttgat taaacttgat taaacttgat taaacttgat taaacttgat
 4501 aaccactcga ctcatcttgt agcttatctt gcttaaaagt agcttatctt gcttaaaagt
 4561 gggccgcctc tgtgaaccat agcgggtgtg agcgggtgtg agcgggtgtg agcgggtgtg
 4621 gagggtacct taaagaaaac aaaactggac agcgggtgtg agcgggtgtg agcgggtgtg
 4681 cttgagtttc tcaaaagcca tccggaagatg cgggaagatg cgggaagatg cgggaagatg

FIG. 4B-3

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4741 gtggattttt gtggctgggt tttctgaagt ctgaggaaca atgccttaag aaaaaacaaa
 4801 cagcaggaat cgtgaggaca gtttcctgtg gccagccgag cctggcagtg ctggcacccg
 4861 gagctggcct gacgcctcaa gacggggcac gacccgtcat ctccggggcc aggggctgca
 4921 gcccgccggt cctgttttg ccttatgtgt gtttaagaaa aatggaggta gtccaaaaa
 4981 agtggcaaat cccgttggag gttttgaagt ccaacaaatt taaacgaat ccaaagtgtt
 5041 ctcacacgtc acatacgatt gagcatctcc atctggtcgt gaagcatgtg gtaggcacac
 5101 ttgcagtggt acgacggaa tgctttttat taagaagcaag tagcatgaag tattgcttaa
 5161 attttaggta taaataaata tatatatgta taatatatat tccaatgtat tccaagctaa
 5221 gaaacttact tgattccttat gaaatcttga taaaatatatt ataatgcatt tatagaaaaa
 5281 gtatatatat atataaaaa tgaatgcaga ttgcgaagggt ccctgcaaat ggatggcttg
 5341 tgaatttgct ctcaagggtc ttatggaaag ggcacacat tgcctccgtg tcatgttttc
 5401 tcaagctcca gattggctag atttcagatc gccaacacat tcgccaactgg gcaactaccc
 5461 tacaagtctg tactttcatt ttaattatatt tctaacagaa ccgctccgt ctccaagcct
 5521 tcatgcacat atgtacctaa tgagttttta tagcaaaagaa tataaatgtg ttttacattt
 5561 ttgtatgaat tttttcacia aaagatcctg aataagcatt gttttatgaa ttttacattt
 5641 ttcctcaccia tttagcaatt ttccgaatgg taataatgtc taaatctttt tcctttctga
 5701 attcttgctt gtacattttt ttttaccttt caaaggtttt taattatttt tgtttttatt
 5761 ttgtacgat gagttttctg cagcgtacag aattgttgc tgcagattct attttcagaa
 5821 agtgagagga gggaccgtag gtcttttcgg agtgacacca agattgtgt ctttcctggt
 5881 ctgtcctagg agctgtataa agaagcccag gggctctttt taactttcaa cactagtagt
 5941 attacgaggg gtggtgtgtt ttccctctcc gtggcaagggt cagggagggt tgcttaggat
 6001 gcccgccac cctgggaggc ttgccagatg ccggggggcag tcagcattaa tgaaactcat
 6061 gtttaaaact ctctgaccac atcgtcagga tagaatctta acttgagttt tccaaacacc
 6121 ttttgagcat gtcagcaatg catggggcac acgtgggggt tggctgcaag gaaccagggt
 6181 ccactgcagc cacgtggcca gccctggatt ttggagcctg ggtatgatg cctgaccagg
 6241 acccttgctg cctggtgaac ctgcaggagg ggtatgatg cctgaccagg acagccagtc
 6301 ttactctttt ttctcttcaa cagtaactga cagtcacgtt ttactggtaa cttattttcc
 6361 agcacatgaa gccaccagtt tcatctcaaa gtgtatatgt ggttcagact tgggggcaga
 6421 agttcagaca caccgtgctc agaggggacc agagcccgag cagagccgag ttcggagtt
 6481 tacagggtag ctctgaaat taactcaaac ttttgaccaa atgagtgtag atctctggat
 6541 tcaacttgct actgggctgc tgatggtcag ctctgagaca gtggtttgag agcaggcaga

FIG. 4B-4

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FIG. 4B-5

6601 acggtcttgg gactgttttg actttccctt ccctggtggc cactctttgc tctgaagccc
 6661 agattggcaa gaggagctgg tccattcccc attcattggca cagaacagtg gcagggccca
 6721 gctagcaggc tcttctggcc tccctggcct cattctctgc atagccctct ggggacccctg
 6781 ccacctggcc tcttaccctg ccttggttta aacatggact tgggagggaa tgcattcatc cacttttttt
 6841 ttttaagcag atgatgggat aatgggact atggagacga aaatgtgctg agcctggcag ggggagggcaa
 6901 ttaattctaa tctcattcaa ttagctcact gctcttctcc acagcaagcg tccagggcag atggcagagg
 6961 gtttcattctg tttctttgaa gacacactcg gcgtctgcag tggaccttta tggaccttcat gtgttctccc
 7021 tttctttgaa atctgcctcg tgtgggtcct tggaggtcag cctccttcat aggccttggg ggcactggct
 7081 atctgcctcg tgtgggtcct tggaggtcag cctccttcat aggccttggg ggcactggct
 7141 tgtgggtcct tggaggtcag cctccttcat aggccttggg ggcactggct
 7201 gggagtcagc cctccttggc tctgacctgc cagcaggcgg ccagggctct ttgaaaatct tgcgttaatg gtctgacaca
 7261 cctccttggc cagcaggcgg ccagggctct ttgaaaatct tgcgttaatg gtctgacaca
 7321 cagcaggcgg ccagggctct ttgaaaatct tgcgttaatg gtctgacaca
 7381 cagcaggcgg ccagggctct ttgaaaatct tgcgttaatg gtctgacaca
 7441 tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7501 aaagcaagtt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7561 aacattcctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7621 tgggccccacg tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7681 gaatacatct tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7741 ctttataatg tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7801 gcctttctgt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7861 acaaaaacct tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7921 aaattgtact tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 7981 gaaaaaaagt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8041 gaaacagtgt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8101 tgatttggag tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8161 gccaggcgag tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8221 ggcggactcg tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8281 gtgattgtatg tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8341 caattatact tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt
 8401 ttagctcgcc tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt tttacttctt

SEQ ID NO: 8

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LRQGGTLTGKFMSTSSIPGCLLGVALEGDGSPHGHASLLQHVL
LEQARQQSTLIAVPLHGQSPVLTGERVATSMRTVGKLPRRPLSRTQSSPLPQSPQAL
QQLVMQQHQQFLEKQKQQQLQGLKILTKTGELPRQPTTHPEETEELTEQQEVLLGE
GALTMPREGSTESESTQEDLEEEDEEEDGEEEDCIQVKDEEGESGAEEGPDLEEPGA
GYKKLFSDAQPLQPLQVYQAPLSLATVPHQALGRTQSSPAAPGGMKSPDQPVKHLFT
TGVVYDTFMLKHQCMCGNTHVHPEHAGRIQSIWSRLQETGLLSKCERIRGRKATLDEI
QTVHSEYIHTLLYGTSPNLRQKLDKLLGPI SQKMYAVLPCGGIGVDSDTVWNEMHSS
SAVRMAVGCILLELAFKVAAGELKNGFAIIRPPGHAAEESTAMGFCFFNSVAITAKLLQ
QKLNVGKVLIVDWDIHHGNGTQQAFYNDPSVLYISLHRYDNGNFFPGSGAPEEVGGGP
GVGYNVNVAWTGGVDPPIGDVEYLTAFTVVMPIAHEEFPDVVTLVSAGFDAVEGHLSP
LGGYSVTARCFGLTRQLMTLAGGRVVLALGEGHDLTAICDASEACVSALLSVELQPL
DELVLQKPNINAVATLEKVIETQSKHWSCVQKFAAGLGRSLREAQAGETEEAETVSA
MALLSVGAEQAAAAAREHSPPRAEFPMEQEPAL (SEQ ID NO:9)

FIG. 5A

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```
1  ccctgcgcca  ggtggcacg  ctgaccggca  agttcatgag  cacatcctct  attcctggct
61  gcctgctggg  cgtggcactg  gagggcgacg  ggagccccc  cgggcattgc  tccttgctgc
121 agcatgtgct  gttgctggag  caggcccggc  agcagagcac  cctcattgct  gtgccactcc
181 acggggcagt  ccactagtg  acgggtgaac  gtgtggccac  cagcatgcgg  acggtaggca
241 agctcccgg  gcatcggccc  ctgagccgca  ctcagtcctc  accgctgccg  cagagtcccc
301 agggccctgca  gcagctggtc  atgcaacaac  agcaccagca  gttcctggag  aagcagaagc
361 agcagcagct  acagctgggc  aagatcctca  ccaagacagg  ggagctgccc  aggcagccca
421 ccaccaccc  tgaggagaca  gaggaggagc  tgacggagca  gcaggaggtc  ttgctggggg
481 agggagccct  gaccatgccc  cgggagggct  ccacagagag  tgagagcaca  caggaaagacc
541 tggaggagga  gacgaggaa  gaggatggg  aggaggagga  ggattgcatc  caggttaagg
601 acgaggaggg  cgagagtgt  gctgaggagg  ggcccgaatt  ggaggagcct  ggtgctggat
661 acaaaaaact  gttctcagat  gccagccgc  tgagccttt  gcaggtgtac  caggcgcccc
721 tcagccctgg  cactgtgccc  caccaggccc  tgggccgtac  ccagtcctcc  cctgctgccc
781 ctgggggcat  gaagagcccc  ccagaccagg  ccgtcaagca  cctcttcacc  acaggtgtgg
841 tctacgacac  gttcatgcta  aagcaccagt  gcatgtgcgg  gaacacacac  gtgcaccctg
```

FIG. 5B-1

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901 agcatgctgg ccggtaccag agcatctggt cccggctgca ggagacagggc ctgcttagca
961 agtgcgagcg gatccgaggt cgcaaaagcca cgctagatga gatccagaca gtgcactctg
1021 aataccacac cctgctctac gggaccagtc cctcaaccg gcagaagcta gacagcaaga
1081 agttgctcgg cccatcagc cagaagatgt atgctgtgct gccttgtagg ggcacgagg
1141 tggacagtga caccgtgtgg aatgagatgc actcctccag tgctgtgcgt atggcagtgg
1201 gctgcctgct ggagctggcc ttcaaggtag ctgcaggaga gctcaagaat ggatttgcca
1261 tcatccggcc ccaggacac caccggagg aatccacagc cacgggattc tgcttcttca
1321 actctgtagc catcacgca aaactcctac agcagaagtt gaacgtgggc aaggtcctca
1381 tcgtggactg ggacattcac catggcaatg gcacccagca ggcgttctat aatgaccct
1441 ctgtgctcta catctctctg catcgctatg acaacgggaa ctctttcca ggctctgggg
1501 ctccctgaaga ggttggtgga ggaccaggcg tggggtacaa tgtgaacgtg gcatggacag
1561 gaggtgtgga cccccatt ggagacgtgg agtaccttac agccttcagg acagtggtag
1621 tgccattgc ccacgagttc tcacctgatg tggctcctagt ctccgccggg tttgatgctg
1681 ttgaaggaca tctgtctcct ctgggtggct actctgtcac cgccagatgt tttggccact
1741 tgaccaggca gctgatgacc ctggcagggg gccgggcttg gctggccctg gagggagggc
1801 atgacttgac cgccatctgt gatgcctctg aggttgtgt ctgggctctg ctcagtgtag
1861 agctgcagcc ctgggatgag gcagtcttgc agcaaaagcc caacatcaac gcagtggcca
1921 cgctagagaa agtcatcgag atccagagca aacctggag ctgtgtgcag aagttcgccg
1981 ctggtctggg ccggtccctg cgagaggccc aagcaggtga ggcgaggag gccgagactg
2041 tgagcgccat ggccttgctg tcggtggggg ccgagcaggc ccaggctgcg gcagcccggg
2101 aacacagccc caggccggca gaggagccca tggagcagga gcctgccctg tgacgccccg
2161 gccccatcc ctctcggctt caccattgtg atttgttta tttttctat taaaaacaaa
2221 aagtcacaca ttc (SEQ ID NO:10)

FIG. 5B-2

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1 mtstgqdstt trqrrsrqnp qspqqdssvt skrnikkjav prsipnlaev kkkgkukklg
 61 gameedliiv lqgmdlnlea ealagtglvl deqlnefhcl wddsfpegpe rlhaikqgli
 121 qeglldrcvs fqarfaeke lmlvhsleyi dlmettqymn egelrvladt ydsvylhpn
 181 yscaclasgs vlrlvdavlg aeirngmai rppghahqs lmdgycmfhn vavaaryaqg
 241 khrirrvliv dwdvhgggt qftfdqdpv lyfsihryeq grfwphlks nwsttgfgqg
 301 qgytinvpwn qvgmrdadyi aafhlvllpv alefqpqlvl vaagfdalqg dpkgemaatp
 361 agfaqlthll mglaggklil sleggnira laegvsaslh tllgdpcpml espgapcrsa
 421 qasvscalea lepfwevlvr stetverdnm eednveese eggweppvlp iltwplqsr
 481 tglvydqnmn nhcnlwdshh pevpqrilri morleelgia grcltitprp ateaelltch
 541 saeyvghlra tekmtrelh ressnfdsiy icpstfacaq latgaacrly eavisgevin
 601 gaavvrppgh haeqdaacgf cffnsavavaa rhaqtisgha lrilivdwdv hhngngtghmf
 661 eddpsvlyvs lhrydhgtff pmgdegassq igraagtgt vnvaungprm gdadylaawh
 721 rlvlpiaef npelvlvsag fdaargdplg gcqvspegia hlthllmgl sgrilileg
 781 gynltsises maactrsilg dppplltlpr pplsgalasi tetiqvhrry wrslrvmkve
 841 dregpssskl vtckapqpak prlaermtrr ekkvleagmg kvtsasfgee stpgqtnset
 901 avvalcqddp seaatggatl aqtiseaaig gamlgqttse eavggatp dq ttseetvga
 961 ildqttse da vggatigqtt seeavggatl aqtiseaame gatldqttse eapggtelig
 1021 tplasstdhq tpptspvqgt tpqispstli gslrtlelgs esqgasesqa pgeenllgea
 1081 agggqmadsm lmqgsrgltd qaifyavtpl pwcphlvavc pipaagldvt qpcgdcgtiq
 1141 enwvclscyq vycgryingh mlqhhgnsgh plvlsvydlis awcyycqayv hhqalldvkn
 1201 iahqnkfged mphph (SEQ ID:11)

FIG. 6A

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```
1  gggcagtccc  ctgaggagcg  gggctggttg  aaacgctagg  ggcgggatct  ggcggagtgg
61  aagaaccgcg  gcaggggcca  agcctcctca  actatgacct  caaccggcca  ggattccacc
121  acaaccaggc  agcgaagaag  taggcagaac  cccagtcgc  ccctcagga  ctccagtgtc
181  acttcgaagc  gaaatatata  aaagggagcc  gttccccgct  ctatcccaa  tctagcggag
241  gtaagaaga  aaggcaaat  gaagaagctc  ggccaagcaa  tggaagaaga  cctaatacgtg
301  ggaactgcaag  ggatggatct  gaacctcgag  gctgaagcac  tggcttgggtg  ggaaggccct
361  ttggatgagc  agttaaata  attccattgc  ctctgggatg  acagcttccc  gcctcctaga
421  gagcggctcc  atgccatcaa  ggagcaactg  atccaggagg  gcctcctaga  tcgctgcgtg
481  tcctttcagg  cccggtttgc  tgaaaaggaa  gagctgatgt  tggttcacag  cctagaatat
```

FIG. 6B-1

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541 attgacctga tggaacaac ccagtacatg aatgaggagg aactccgtgt ctagcagac
601 acccacgact cagtttatct gcattccgaac tcatactcct gtgcctgcct ggctcaggg
661 tctgtcctca ggctggtgga tgcggtcctg ggggctgaga tccggaacgg catggccatc
721 attaggcctc ctggacatca cggccagcac agtcttatgg atggctattg catgttcaac
781 cacgtggctg tggcagcccg ctatgctcaa cagaaacacc gcaccggag ggtccttacc
841 gtagattggg atgtgcacca cggtaagga acacagttca cttcgacca ggacccagt
901 gtcctctatt tctccatcca cgcctacgag cagggtaggt tctggccca cctgaaggcc
961 tctaactggt ccaccacagg ttctggccaa ggccaaagat ataccatcaa tgtgccttgg
1021 aaccagggtg ggaatgcgga tgcctgactac attgctgctt tcctgcacgt cctgctgcca
1081 gtcgccctcg agctccagcc tcagctggtc ctggtggccg ctggattga tgcctgcaa
1141 ggggacccca agggcgagat ggcggccact cggcgagggt tgcgccagct aaccacctg
1201 ctcatgggtc tggcaggagg caagctgac ctgtctctgg agggaggcta caacctccgc
1261 gccctggctg aaggcgtcag tgcttcgctc cacaccttc tgggagaccc ttgccccatg
1321 ccggagtcac ctggtgcccc ctgcccggag gccaggctt cagtttccctg tgctctggaa
1381 gcccttgagc cctctggga ggttcttggt agatcaactg agaccgtgga gagggacaac
1441 atggaggagg acaatgtaga ggagagcgag gaggaaaggac cctgggagcc cctgtgctc
1501 ccaatccctga calggccagt gctacagtct cgcacagggc tggtctatga ccaaatatg
1561 atgaatcact gcaacttgtg gcaacttgtg gacagccac caccctgagg tactctgagg
1621 atcatgtgcc gtctggagga gctgggccc tgcagtgctg caccctgagg gacacggcg
1681 cctgccacag aggtgagct gctcaccctg caccgtgaga cagggtgagg agtacgtggg
1741 gccacagaga aaatgaaaac ccgggagctg caccgtgaga tcccaactt tgactccatc
1801 tatactgcc ccagtacctt cgcctgtgca cagcttgcca ctggcgctgc ctgcccctg
1861 gtggaggctg tgctctcagg agaggtcctg aatgggtgctg ctgtggtgctg tcccaggga
1921 caccacgcag agcaggatgc agcttgcggt ttttgctttt tcaactctgt ggctgtggct
1981 gctcgccatg cccagactat cagtgggcat gcccacgga tctgagttgt ggattgggat
2041 gtccaccacg gtaatggaac tcagcacatg tttgaggatg acccagttgt gctatatgtg
2101 tccctgcacc gctatgatca tggcaccttc tcccacatgg gggatgaggg tccagcagc
2161 cagatcggcc gggccgcggg cacaggcttc accgtcaacg tggcatggaa cgggccccgc
2221 atgggtgatg ctgactacct agctgcccgg cctgcctgg tgcttcccat tgcctacgag
2281 ttaaccacg aactgggtgt ggtctcagct ggctttgatg ctgcacgggg ggtaccgctg

FIG. 6B-2

2341 gggggctgcc aggtgtcacc tgagggttat gccacctca ccaacctgct gatgggcctt
 2401 gccagtggcc gcattatcct taccctagag ggtggctata acctgacatc catctcagag
 2461 tccatggctg cctgcactcg ctccctcctt ggagacctcac atcactgaga caccctgctt gaccctgcca
 2521 cggccccccac tatcaggggc cctggcctca cactgagag aaggaccttc ccatccaagt ccatcgaga
 2581 tactggcgca gcttacgggt catgaaggca gaagacagag aagaccttc tagctgagcg cagttctaag
 2641 ttggtcacca agaaggcacc ccaaccagcc aaacctaggt taagacctc gatgaccaca
 2701 cgagaaaaga aggttctgga agcaggcatg ggaaagtca cctcgcatc atttgggga
 2761 ggtccactc caggccagac taactcagag acagctgtgg tggccctcac tcaggaccag
 2821 ccctcagagg cagccacagg ggagccact ctggcccaga ccatcttga ggcagccatt
 2881 gggggagcca tgctgggcca gaccacctca gaggaggctg tcggggggagc cactccggac
 2941 cagaccacct cagaggagac tgtgggagga gccattctgg accagaccac ctcagaggat
 3001 gctgttgggg gagccacgct ggccagact acctcagagg aggtgtagg aggtctaca
 3061 ctggcccaga ccatctcggg ggcagccatg gaggagcca cactggacca gactacgtca
 3121 gaggaggctc cagggggcac cgagctgac caaactcctc tagcctcag cacagaccac
 3181 cagaccccc caacctcac tgtgcaggga actacacccc agatatctcc cagtacactg
 3241 attgggagtc tcaggacctt ggagctaggc agcgaacctc agggggcctc agaatctcag
 3301 gccccaggag aggaagaacct accaggagag gcagctggag gtcaggacat ggctgattcg
 3361 atgctgacgc agggatctag gggcctcact gatcaggcca tattttatgc tgtgacacca
 3421 ctgccctgggt gtcccattc ggtggcagta tgcccatac ctgcagcagg cctagacgtg
 3481 acccaacctt gtggggactg tggaacaatc caagagaact ccaatgctcc aatctctgga
 3541 caggtctacc gtggtcgtta catcaatggc cacatgctcc aacacctgg gttactactg
 3601 caccgcctgg tcctcagcca catcgacctg tcagcctggc gttactactg tcaggcctat
 3661 gtccaccacc aggtctctct agatgtgaag aacatcgccc accagaaaca gtttggggag
 3721 gatattgccc acccacata agcccagaa tacggtccct ctccacctc tgaggcccac
 3781 gatagaccag ttccagcctg ttccaggctg taccttggat gaggggtagc ctccactgc
 3841 atcccatcct gaatatcctt tgcaactccc caagagtgtc tatttaagtg ttaatacttt
 3901 taagagaact gcgacgatta attgtggatc tccccctgcc catcgcccgc ttgaggggca
 3961 cactactccc agccagaaag gaaagggggg cagctcagtg gcccagaag ggagccgata
 4021 tcatgaggat aacattggcg ggaggggagt taactggcag gcattggcaag gttgcatatg
 4081 taataaagta caagctgtt (SEQ ID NO: 12)

FIG. 6B-3

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1  mdlrvqgrpp  vepppeptll  alqrpqrlhh  hlflaglqqq  rsvepmrlsm  dtpmpelqvg
61  pgeqelrqll  hkdkskrsav  assvkvkqla  evilkkgaa  lertvhpns  gipyrtlepi
121  etegatrsm1  ssflppvpsi  psdppehfpl  rktvsepnk  lrykpkksle  rrknpllrke
181  sappslrrrp  aetlgdssps  ssstpasgcs  spndsehgn  pilgdsdrrt  hptlgprgpi
241  lgsphthplf1  phglepeagg  clpsrlqpil  lldpsgshap  lltvpglgpl  pfhfaqsimt
301  terlsqsglh  wplsrtrsep  lppsatappp  pgpmqprleq  lkthvqvkr  sakpsekprl
361  rqipseaedle  tdgggpgqv  ddglehrelg  hgqpearpa  plqqhqvii  weqqlagrl
421  prgstgdcvi  lplaggghrp  lsraqsspa  pasisapepa  sqarvlsse  tpartlpflt
481  gliydsvmlk  hqcscgdnsr  hpehagriqs  iwsrlqergl  rsqceclgr  kasieelqsv
541  hserhvllyg  tnplsrlkld  ngklagiliaq  rmfemlpcgg  vgvtdtdtiwn  elhssnaarw
601  aagsvtdlaf  kvasrelkng  favvrppghh  adhstamgfc  ffnsvalacr  qlqqqskask
661  askilivdwd  vhhngtqqt  fyqdpvlyi  slhrhddgnf  fpqsgavdev  gagsgegfnv
721  nvawagglbp  pmgdpeylaa  frivvmpiar  efsdpdlvls  agfdaaeghp  aplggyhvsa
781  kcfgymtqq1  mnlaggavvl  alegghdlt  icdaseacva  allgnrvdpl  seegwkkqpq
841  pqchplsgr  dpqaq (SEQ ID NO:13)

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FIG. 7A

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1 ataataccta ccttgccagga ccacgacagg attaagtggag gaaaaacccc catgagagtg
 61 ttttgccatt gtcaagtggag cctgaggggag gctgagggggg gatcaggctg tatcatgccc
 121 ccgaggacaa actttccagt ttaccctgct agcccatgga cctgcgggtg gtccctaggc tgcccaggc
 181 cctgcgacaga cacaccaggc cctcagccgc gagccacat tgctggccct ggtggagccc atgaggctct
 241 cccagtgga gcccaccaa ggcctgcagc agcagcgtc ggaacaaagag ctgcggcagc
 301 accaccact ctccctagca ggcctgcagc agcagcgtc cagcgtggtc aagcagaagc
 361 ccatggacac gccgacgcc gagttgcagg tggacccca ctgtagccag cagcgtggtc catcccaaca
 421 ttctccaaa ggaacagagc aagcgaagtg aagcgtggtc cctggagagc ggaacaaagag ctgcggcagc
 481 tagcggaggt gattctgaaa aaacagcagg cggccctaga aagaacagtc aagcagaagc
 541 gcccgggcat tccctacaga acccgggagc cctggagagc ggaagggagc accgctcca
 601 tgctcagcag ccttcggcct cctgctccca gccgcccag tgacccccc cagcgtggtc aagcagaagc
 661 ctctgcgcaa gacagtctct gagcccaacc tgaagctgag cccgcccag cagcgtggtc aagcagaagc
 721 cggagcggag gaagaatcca ctgctccgaa agcagagtgc gcccaccag cctgggagagc agcagagtgc
 781 gcccgcgaga gacctcgga gacctctccc caagttagtag cagcagccc cctgggagagc agcagagtgc
 841 gcagtcccc caatgacagc gagcacggcc ccaatcccat cctgggagagc cctgggagagc agcagagtgc
 901 gacccatcc gactctgggc cccggggggc caatccctggg gagcccccac actcccctct
 961 tctgcccga tggcttggag cccgaggctg ggggacactt gccctcccgc ctgcagccca
 1021 ttcctctctt ggacccctca ggctctcatg cccgctgct tgaagctgct gactgtgccc gggcttgggc
 1081 ccttgccctt ccaacttgcc cagtccttaa tgaccaccca ggggtctctt ggggtcaggcc
 1141 tccactggcc actgagccgg actcgtcag agccctgccc cccagtgcc accgctccc
 1201 caccgcccgg cccatgcag cccgcccctgg agcagctcaa aactcacgtc caggtgatca
 1261 agaggtcagc caagccgagt gagaagcccc ggtgcggca gataccctcg gctgaagacc
 1321 tggagacaga tggcggggga ccgggcccagg tggtagcga cggcccggag cacaggagac

FIG. 7B-1

1381 tgggccatgg gcagcccgag gccagaggcc ccgctcctct ccagcagcac cctcaggtgt
 1441 tgctctggga acagcagcga ctggctgggc ggctcccccg gggcagcacc ggggacactg
 1501 tgctgcttcc tctggcccg ggtgggcacc ggctcttgtc cggggtcag tcttccccag
 1561 ccgcacctgc ctcaactgtca gccccagagc ctgccagcca ctgccagctc ctctccagct
 1621 cagagacccc tgccaggacc ctgcccttca ccacagggtt gatctatgac tcggtcatgc
 1681 tgaagcacca gtgctcctgc ggtgacaaca gcaggcaccc ggagcagcgc gcccgcatcc
 1741 agagcatctg gtcccggttg caggagcggg gagctgcagt cggctcagg ggctctccgag
 1801 gccggaaggc ctccctggaa gctcctcagg cggctcagg ggctcagg ccagtgtgag tgtctccgag
 1861 acggcaccaa ccgctcagg cgcctcaaac gagctgcagt cggctcagg tgaagcggcac gtgctcctct
 1921 cacagcggat gtttgagatg ctgccctgtg cgtccctcagg gaagctggca gggctcctgg
 1981 ggaatgagct tcattccgcc aatgcagccc gctggggttg ggtggacact ggtggacact gacacatct
 2041 ccttcaaagt ggcttctcgt gagctaaaga atggttccg cgtgggcccgc actgacctcg
 2101 accatgcaga tcattcaaca gccatgggct tctgcttctt caactcagtg gccatcgcct
 2161 gccggcagct gcaacagcag agcaaggcca gcaaggccag aaaccttcta caagacccc agtgtgctct
 2221 gggacgtgca ccattggcaac ggcacccagg gacgacggca acttcttccc gggagtgagg gctgtggatg
 2281 acatctccct gcatcgccat tggcagcggc gagggcttca atgtcaatgt ggcctgggct ggaggtctgg
 2341 aggtaggggc acccccccac gggggatcct gactacctgg ctgctttcag gatagtcgtg acgcccatcg
 2401 acccccccac cccgagagt ctctccagac ctagtccctgg tgtctgccgg atttgatgct gctgagggtc
 2461 acccgagagt acccgggccc acggggtggc taccatgttt ctgccaaatg tgctggatg atgacgcagc
 2521 aactgatgaa cctggcagga ggcgcatggg tgctggcctt tgctggcctt catgacctca
 2581 cagccatctg tgacgcctct gaggcctgtg tggctgctct cccaacctca atgcccactg aggtggatc
 2641 ccctttcaga agaaggcttg taaatactgg ggctgcatgc agcgccctgg ctcctctggag
 2701 gccgtgatcc ggtgacacag tgcctagagt gccaggggct gacaaagaag aagtggaggc agtgaccgca
 2761 gactccctggg tgcctagagt gccaggggct gacaaagaag aagtggaggc cctcggagca gctgggtggag
 2821 ctggcgtccc tctctgtggg catcctggct gaagataggc ggaacatctt gcccggcccac catgcccttg
 2881 gaggaagaac ctatgaatct ctaaggctctt ggaacatctt agccccatt cctgggtctt tagagatcct
 2941 ggacctgggtt ctcttctaac ccctggcaat agccccatt cctgggtctt tgacagtatt tgacagtatt
 3001 gtgggcaagt agttgggaac agagaacagc ctgcccctgtt tgacagtatt tgacagtatt
 3061 gtgggcaagt agttgggaac agagaacagc ctgcccctgtt tgacagtatt tgacagtatt
 3121 gtgagaaaaat c (SEQ ID NO:14)

FIG. 7B-2

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1 meepeepads gqslvpvviy speyvsmcde lakipkrasm vhsleayal hkqmrivkpk
61 vasmeeemaf htdaylqhlq kvsqegdddh pdsieyglgy dcpategifd yaaaiggati
121 taaqclidgm ckvainwsgg whhakkdeas gfcylndavl gilrlrrkfe rilyvdlldlh
181 hgdgvedafs ftskvmvtsl hkfspgffpg tgdvsdvglg kgryysvnpv iqdgigdeky
241 yqicesvlke vyqafnpkav vlqlgadtia gdpmcfsnmt pvgigkclky ilqgwlatli
301 lggggynlan tarcwtyltg vilgktlisse ipdbefftay gpdvyleitp scrpdrneph
361 riqqilnyik gnlkhvv (SEQ ID NO:15)

FIG. 8A

1 gaaattcggc acgagctcgt gccgaattcg gcacgagaac ggtttaagc ggaagatgga
61 ggagccggag gaaccggcgg acagtgggca gtcgctggtc ccggtttata tctatagtc
121 cgagtatgtc agtatgtgtg actccctggc caagatcccc aaacgggcca gtatggtgca
181 ttctttgatt gaagcatatg cactgcataa tgatgcttat ctgcagcatc tcaagagtgc
241 ctccatggag gagatggcca ccttccacac atcatccgga ctccatagaa tatgggctag gttatgactg
301 cagccaagag ggcgatgatg taaggatgat ttgactatgc agcagctata ggaggggcta cgatcacagc
361 ccagccact tgcccaatgc ctgattgacg gaatgtgcaa agtagcaatc aactggtctg gaggtggca
421 tgcccaatgc ctgattgacg gaatgtgcaa agtagcaatc aactggtctg gaggtggca
481 tcatgcaaa gacgatgtg aaagatgaag catctgggtt ttggttatctc aatgatgctg tcctgggaat
541 attacgatg cgacggaaat ttgagcgtat tccctacgtg gattcggatc tgcaccatgg
601 agatggtgta gaagacgcat tcagtttcac ctccaaagt ctagcgtgtg ccctgcacaa
661 attctccca gattttttcc caggaacagg tgacgtgtcc gacgttggcc tagggaagg
721 acggtactac agtgtaaatg tgcccatcca ggatggcata caagatgaaa aatattacca
781 gatctgcgaa agtgtactaa aggaagtata ccaagcctt aatcccaag cagtggctct
841 acagctggga gccgacacaa tagctgggga tcccatgtgc tcctttaaca tgaactccagt
901 gggaaattggc aagtgtctca agtacatccc tcaatggcag ttggcaacac tcatttcggg
961 aggaggaggc tataaccttg ccaacacggc tcgattgctg acatacttga ccggggtcat
1021 cctagggaaa acactatcct ctgagatccc agatcatgag tttttcacag catabggtcc
1081 tgattatgtg ctggaatatca cgccaagctg ccggccagac cgcaatgagc ccacccgaat
1141 ccaacaaatc ctcaactaca tccagagctg aggagtgggaa tctgaaagcat gtggtctagt tgacagaaaag
1201 agatcagggt tccagagctg aatgtgtgac tgcaggggaaa atttgaaaga aattacttcc tgaaaatttc
1261 cagtttgrgg caagtggcag taggggaaaga aggagatarc ccaatttaa agttcttatt taataaaaca
1321 caaggggcat caagtggcag taggggaaaga aggagatarc ccaatttaa agttcttatt taataaaaca
1381 caactggacc taggggaaaga aggagatarc ccaatttaa agttcttatt taataaaaca
1441 cacacacaca aatgaaattt ttaatttttg gaaacacagat cagaagctgg atgagagcag tggggagggg
1501 agtattttaa tcatctttaa tgaacacagat cagaagctgg atgagagcag tggggagggg
1561 gtagggcagg aggcagctga caggcagggn tngggcctcn ggaccancca ngtggagccc
1621 tgggagagan ggtactgac ngcagactgg gagg (SEQ ID NO:16)

FIG. 8B

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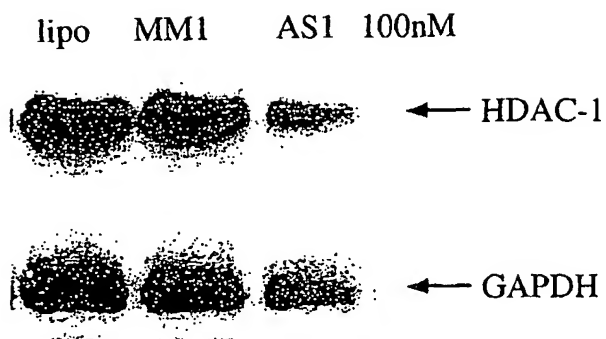


FIG. 9A

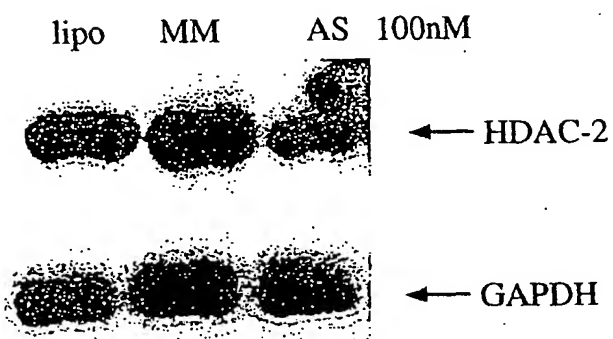


FIG. 9B

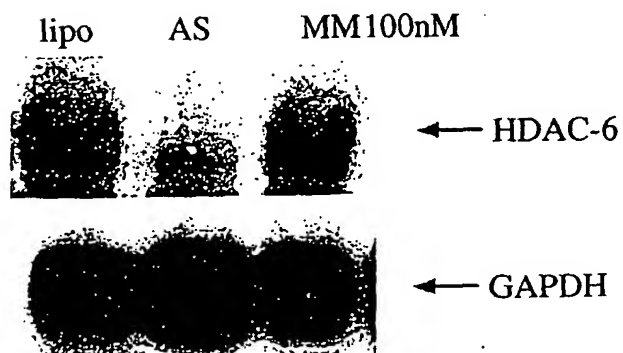


FIG. 9C

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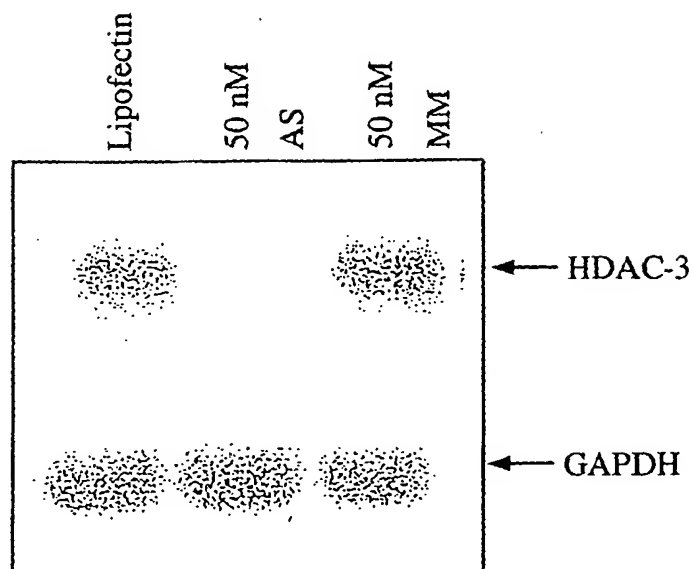


FIG. 9D

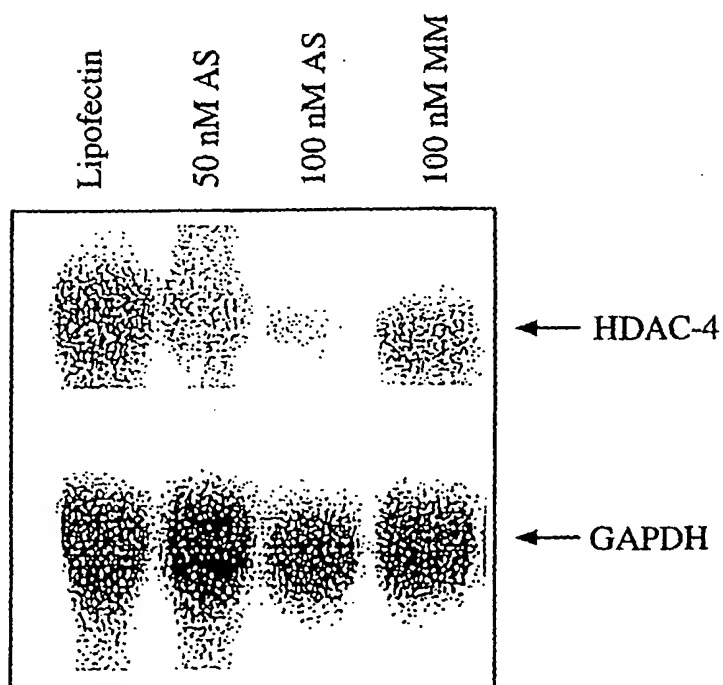


FIG. 9E

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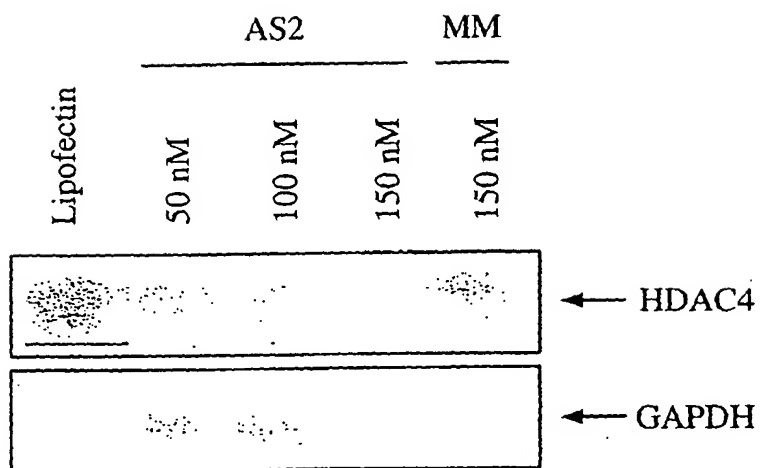


FIG. 9F

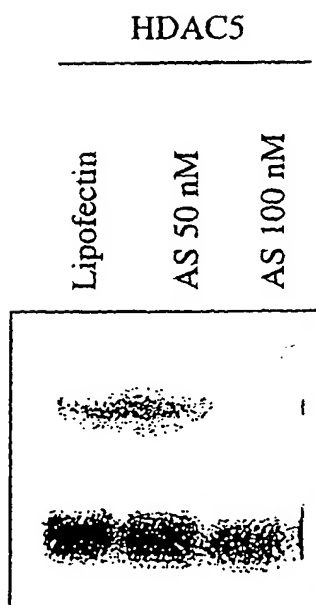


FIG. 9G

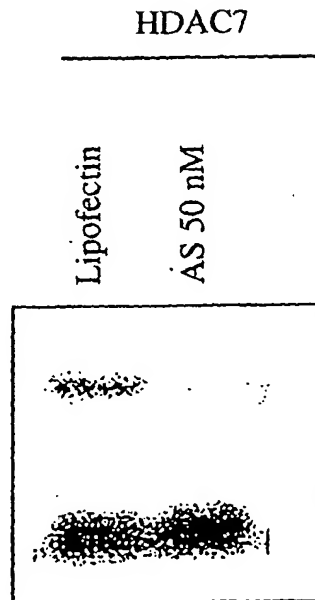


FIG. 9H

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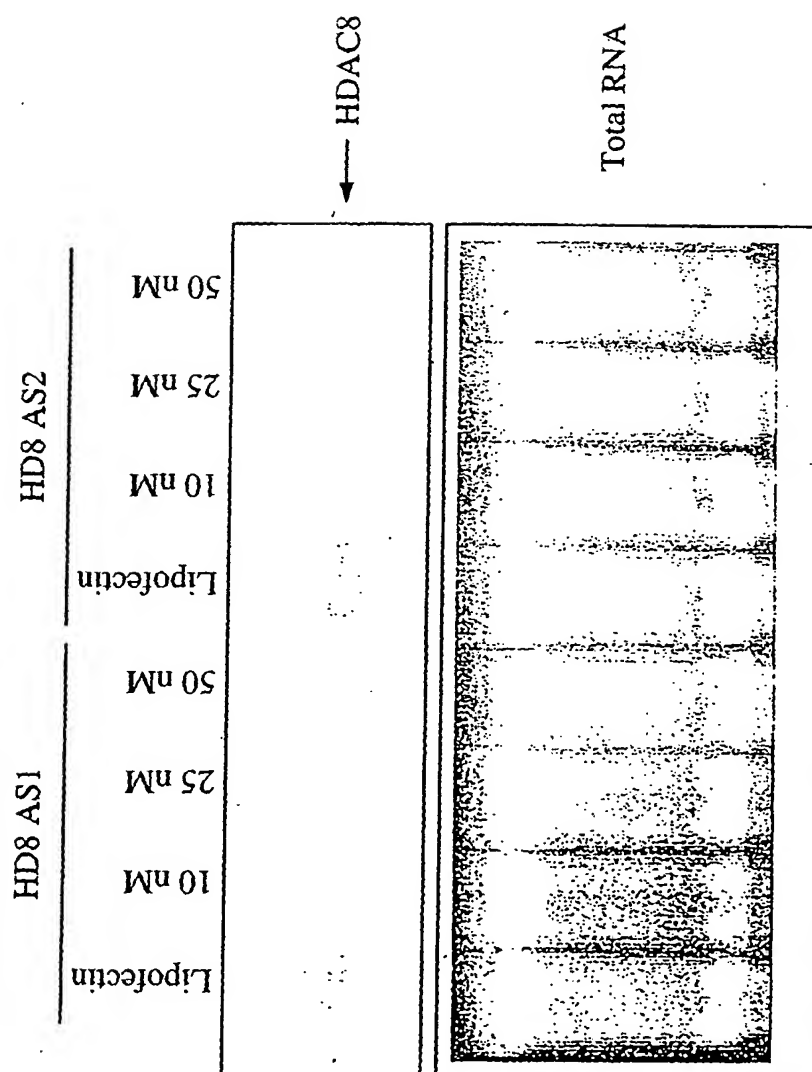


FIG. 9I

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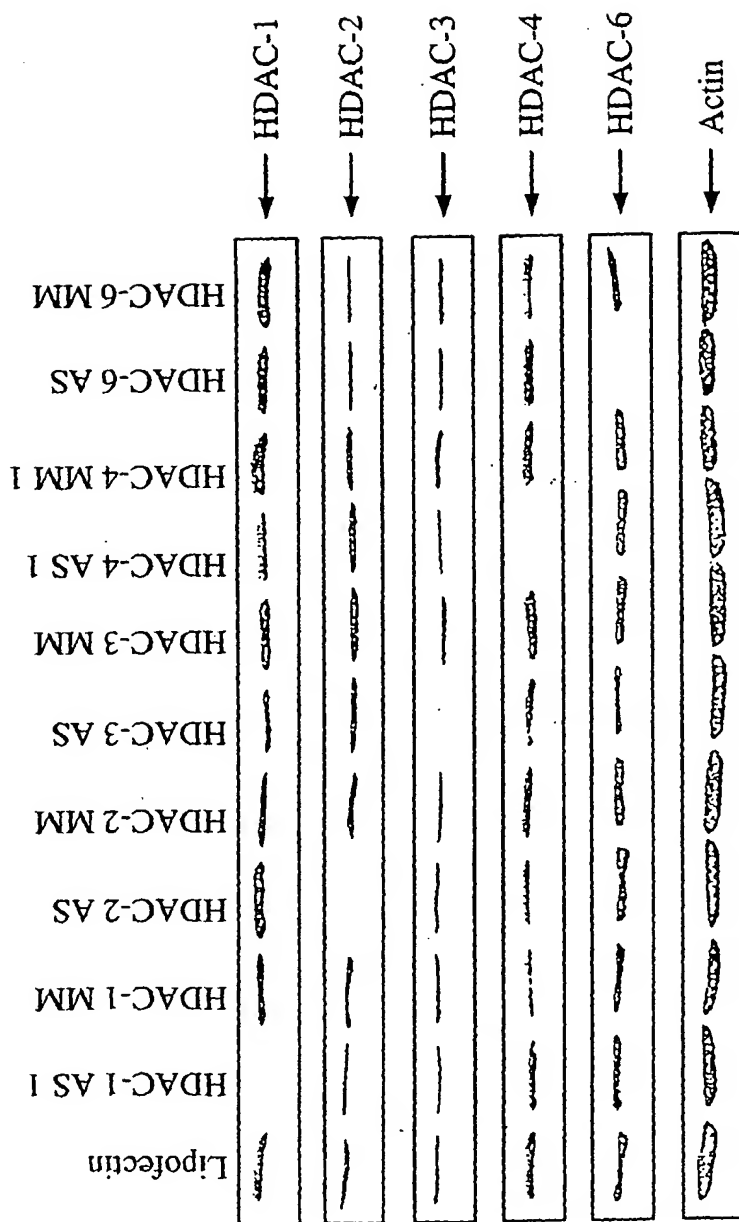


FIG. 10A

AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc - 50nM

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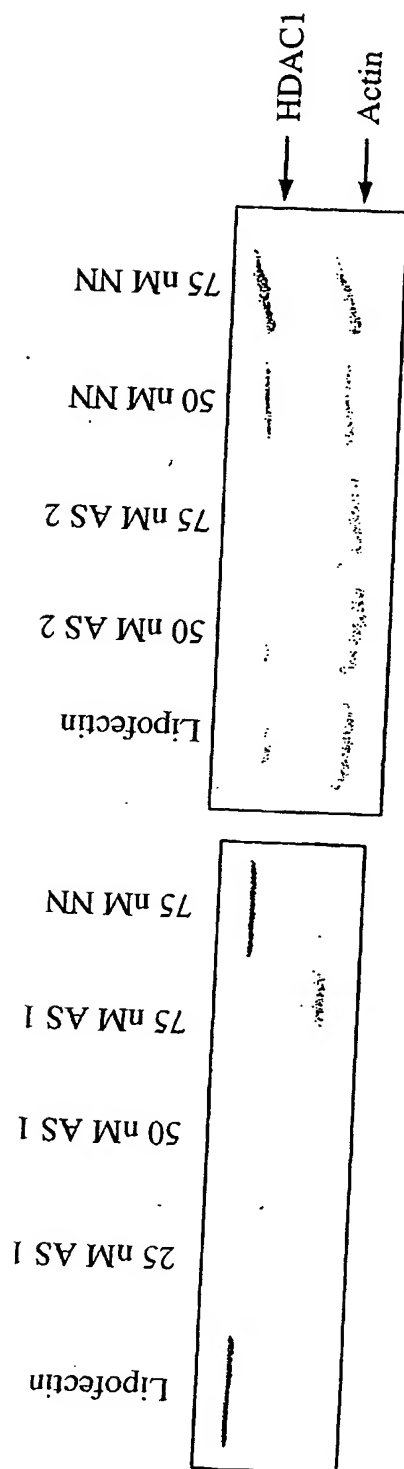


FIG. 10B

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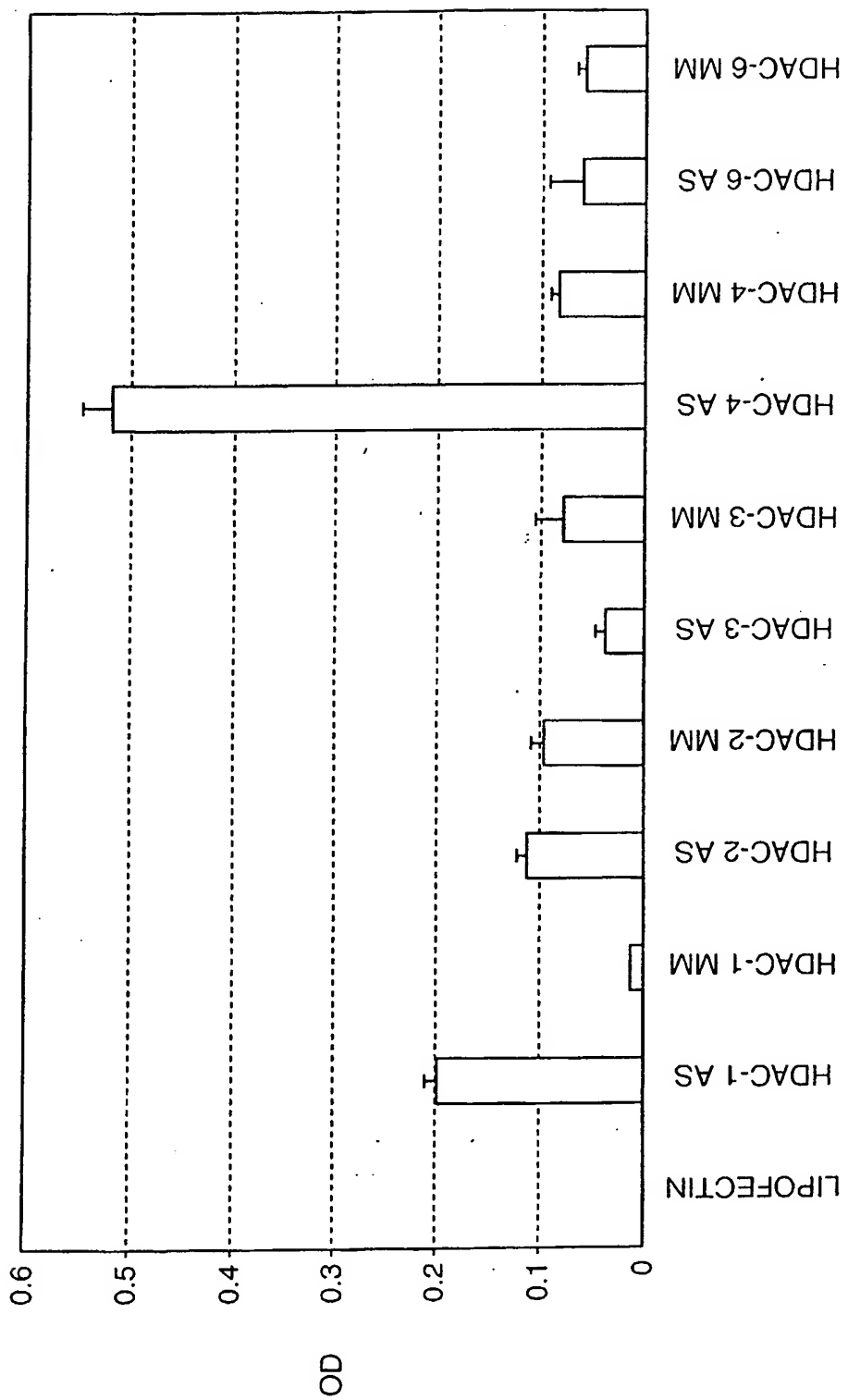


FIG. 11

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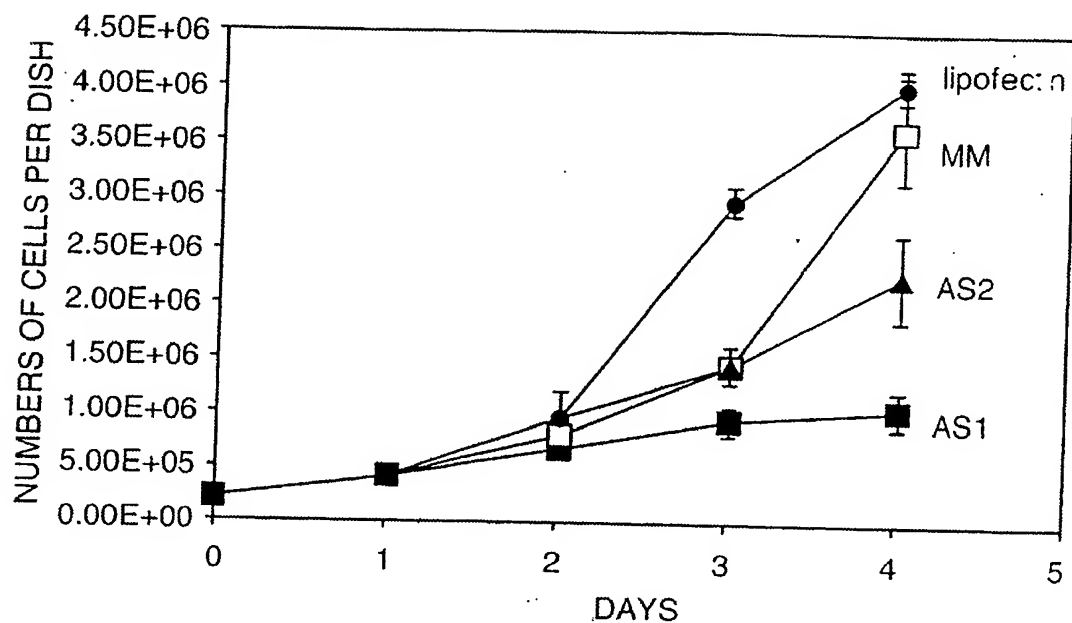


FIG. 12A

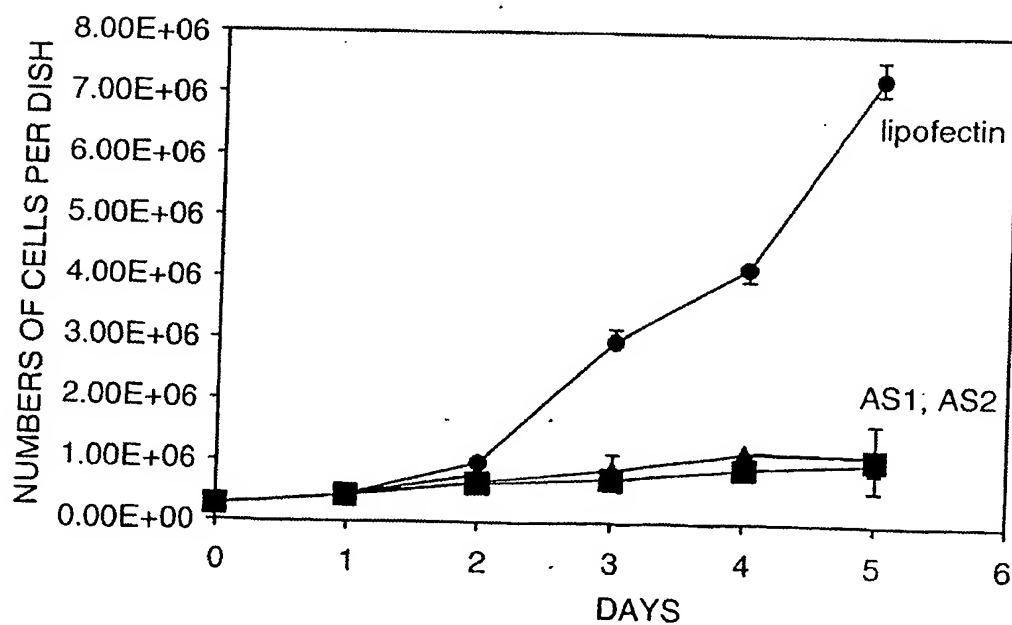


FIG. 12B

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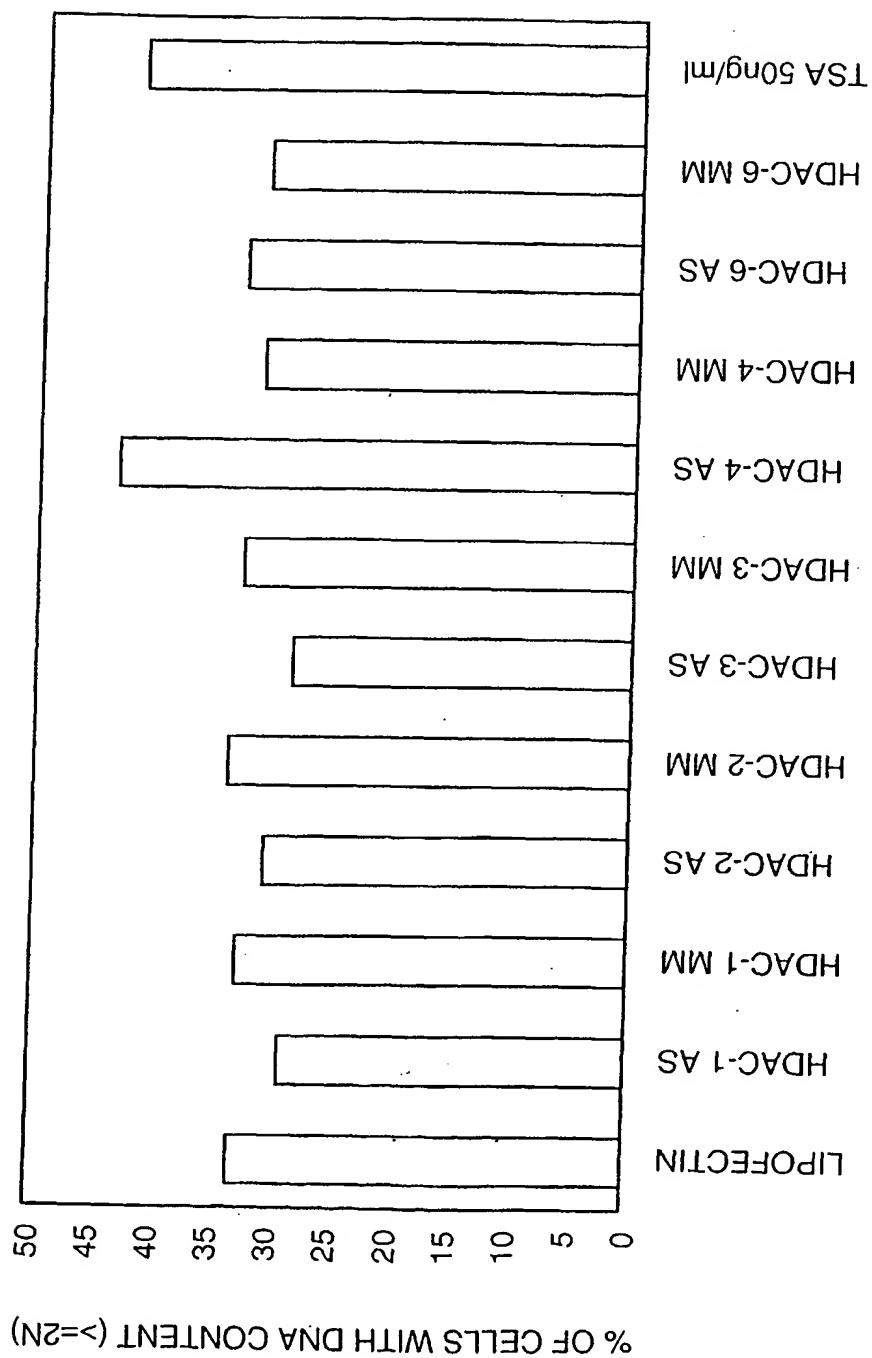


FIG. 13

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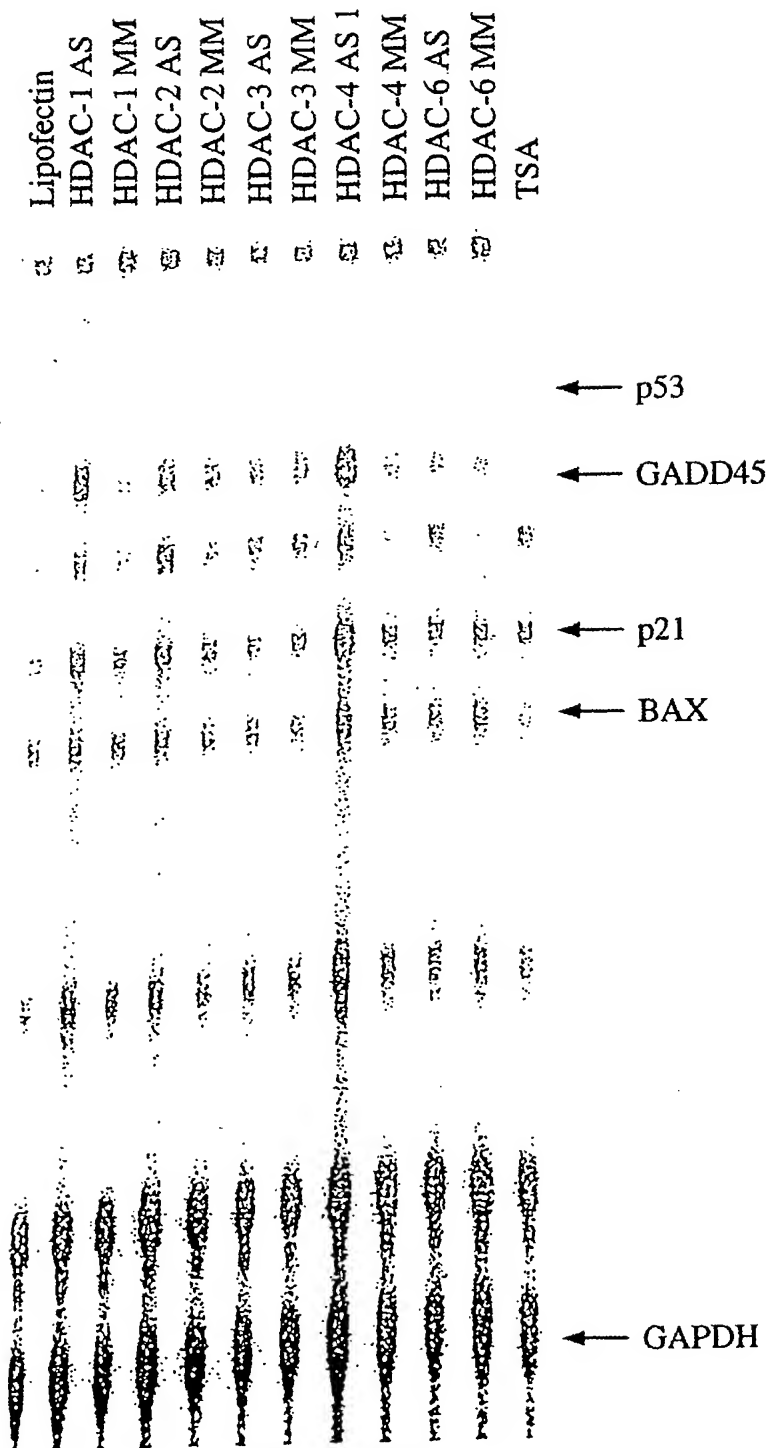


FIG. 14

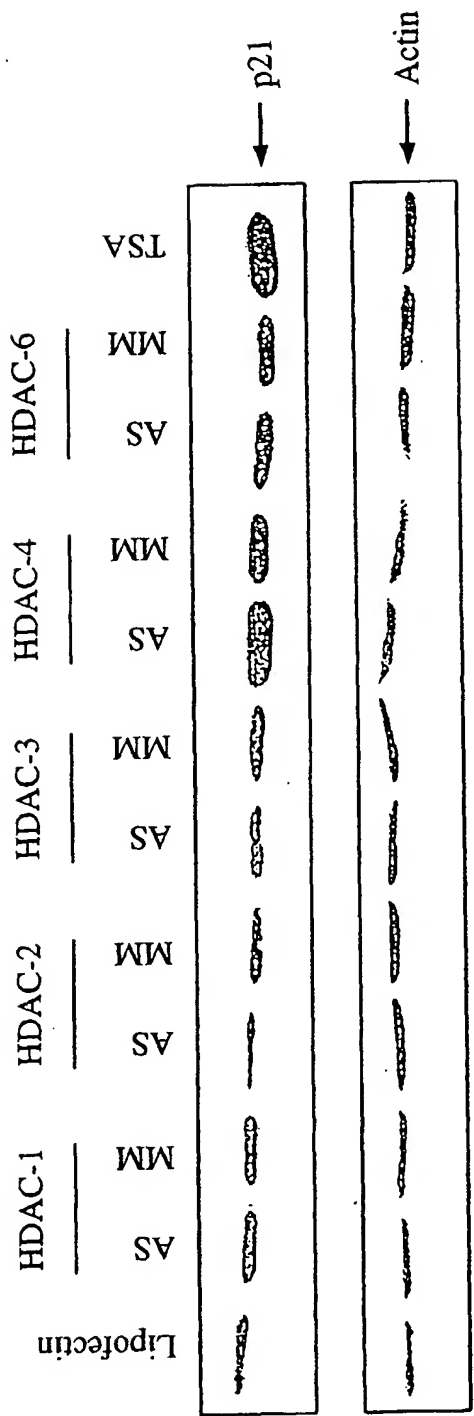


FIG. 15

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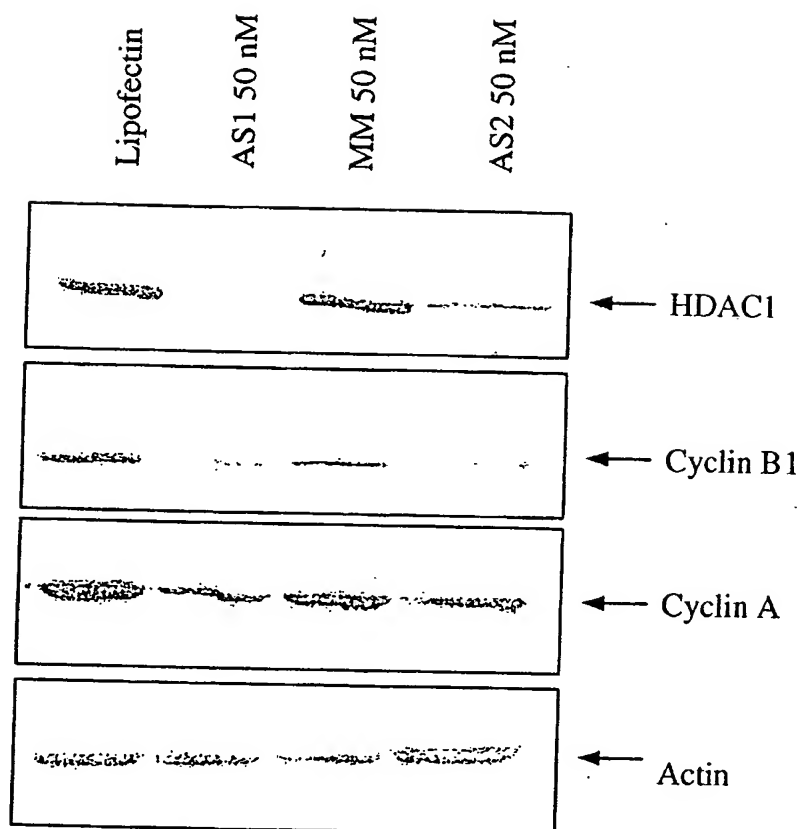


FIG. 16

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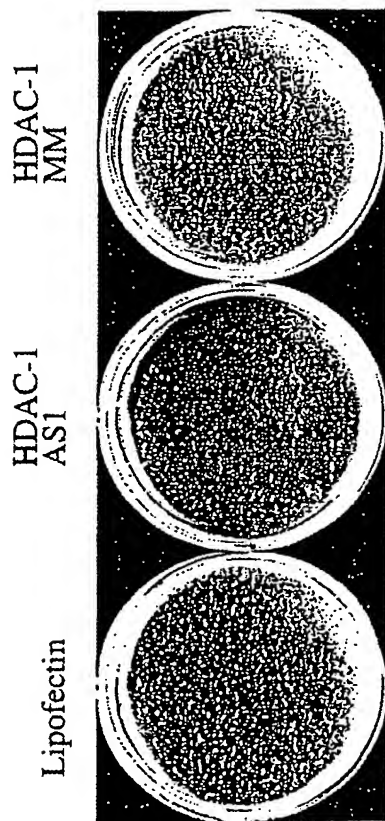


FIG. 17A

-1160

-120

-1200

Colony
Numbers

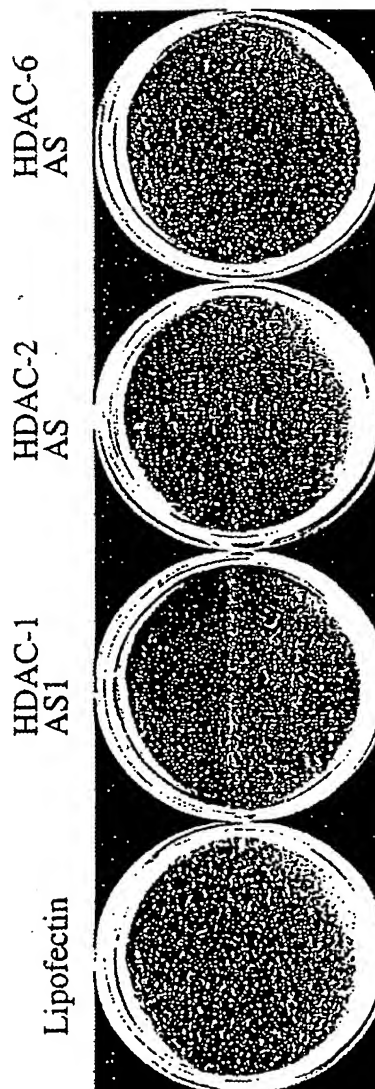


FIG. 17B

-730

-890

-120

-1200

Colony
Numbers

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Organization
International Bureau



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PCT

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A61K 31/7125, C07H 21/04, C12Q 1/44 // A61P 35/00

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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13 May 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2003/006652 A3

(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/11 A61K31/7125 C07H21/04 C12Q1/44 //A61P35/00				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N A61K C07H C12Q				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 97 35990 A (JAMISON TIMOTHY F ;HARVARD COLLEGE (US); TAUNTON JACK (US); HASSIG) 2 October 1997 (1997-10-02) page 5, line 8 -page 6, line 27 page 27, line 13 -page 29, line 2 page 48, line 15 -page 65 claims; examples --- -/-	1-3,6-8, 26-48		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.				
<input checked="" type="checkbox"/> Patent family members are listed in annex.				
* Special categories of cited documents : <table border="0"> <tr> <td> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed </td> <td> *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family </td> </tr> </table>			*A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family
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Date of the actual completion of the international search 28 February 2003		Date of mailing of the international search report 06/03/2003		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Andres, S		

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA M ET AL: "POTENT AND SPECIFIC INHIBITION OF MAMMALIAN HISTONE DEACETYLASE BOTH IN VIVO AND IN VITRO BY TRICHOSTATIN A" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 28, 5 October 1990 (1990-10-05), pages 17174-17179, XP000616087 ISSN: 0021-9258 cited in the application the whole document	1,26,45
A	ZHAO Q ET AL: "EFFECT OF DIFFERENT CHEMICALLY MODIFIED OLIGODEOXYNUCLEOTIDES ON IMMUNE STIMULATION" BIOCHEMICAL PHARMACOLOGY, vol. 51, no. 2, 26 January 1996 (1996-01-26), pages 173-182, XP000610208 ISSN: 0006-2952 the whole document	4,5,9
P,X	WO 00 71703 A (METHYLGENE INC) 30 November 2000 (2000-11-30) the whole document	1-11, 26-48
P,X	WO 00 23112 A (BESTERMAN JEFFREY M ;MACLEOD ALAN ROBERT (CA); METHYLGENE INC (CA)) 27 April 2000 (2000-04-27) examples 9,10 page 29; tables 2,3 claims 38-50	1-12, 26-37, 44-48
E	WO 01 70675 A (METHYLGENE INC) 27 September 2001 (2001-09-27) page 46 -page 54; table 1 page 68; example 13 page 203 -page 223; examples 159-162 claims	1-16, 24-37, 44-48

INTERNATIONAL SEARCH REPORT

international application No.
PCT/IB 01/02907

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 26-33 (as far as in vivo methods are concerned) and claims 34-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 17-23
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9,26-48 (all partially) and claims 10-11

An antisense oligonucleotide against HDAC1; modified forms thereof and its applications in therapy and diagnostic.

2. Claims: 1-9,26-47 (all partially) and claims 12-13

As for subject 1., but concerning HDAC2.

3. Claims: 1-9,26-47 (all partially) and claims 14-15

As for subject 1., but concerning HDAC3.

4. Claims: 1-9,26-48 (all partially) and claim 16

As for subject 1., but concerning HDAC4.

5. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC5.

6. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC6.

7. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC7.

8. Claims: 1-9,26-47 (all partially) and claims 24-25

As for subject 1., but concerning HDAC8.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17-23

The application as filed does not comprise claims 17 to 23. Consequently only claims 1-16 and 24-48 have been taken into account.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 01/02907

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9735990	A	02-10-1997	AU 2990597 A WO 9735990 A2	17-10-1997 02-10-1997
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WO 0023112	A	27-04-2000	AU 6519499 A EP 1243289 A2 EP 1243290 A2 EP 1123111 A1 JP 2002528391 T WO 0023112 A1	08-05-2000 25-09-2002 25-09-2002 16-08-2001 03-09-2002 27-04-2000
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